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# DEFENSE AND PREY USE BY NORTHEASTERN PACIFIC CRYPTOBRANCHIATE DORID NUDIBRANCHS (MOLLUSCA: GASTROPODA)

by

Brian K. Penney

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

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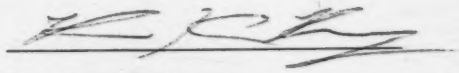

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**Quotes.**

"This has been a beautiful, cool, luminous universe in which to have spent some time. I feel fortunate."

- Donald P. Abbott, Observing Marine Invertebrates

"Write about that. It's what you're talking about, isn't it?"

-Stuart McLean, talking to P. Quarrington, The Boy on the Back of the Turtle

"Chaos reigns within.
Reflect, repent and reboot.
Order shall return."

-Anonymous

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Defense and Prey Use by Northeastern Pacific Cryptobranchiate Dorid Nudibranchs (Mollusca: Gastropoda)," submitted by Brian Keith Penney in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Systematics and Evolution.

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**DEDICATION**

*For my father:*

*Who always knows when the birds return.*

*For my mother:*

*Who wondered aloud at how a hand worked.*

*And for both of them:*

*For letting their son run through the surf*

*Catching minnows in a plastic baggie*

*'Till his lips turned blue.*

## ABSTRACT

Why are some consumers such finicky eaters? For marine consumers, feeding specialization may be partly driven by a need for defense against predators. An ideal system to test this hypothesis is the nudibranchs- colorful, shell-less marine snails that often steal defenses from their prey. Unfortunately, we lack critical data in three areas: a) the importance of defenses other than sequestered chemicals, b) the degree to which nudibranch diets are restricted to prey providing defense, and c) a robust phylogeny against which to test theories of the evolution of feeding specialization. To address these issues, I used cryptobranchiate dorid nudibranchs, a monophyletic taxon that feeds exclusively on sponges.

Defense by reduced nutrient content is common for modular organisms, but is usually thought unavailable to mobile animals. However, I found that nudibranchs contained significantly less organic material per unit wet mass than prosobranch snails, and that differences of similar magnitude deterred the generalist crab, Cancer productus, in laboratory assays. Calcareous spicules, however, were not an effective defense against Cancer crabs, but slightly increased the deterrence of chemical extracts against the anemone Anthopleura elegantissima.

Recent data suggest that most common local dorids prey on a range of sponges: are these slugs "specialized" at all? I compared slug diets to sponge abundance in the field for two nudibranchs, Anisodoris nobilis and Cadlina luteomarginata, at three subtidal sites in Barkely Sound, British Columbia. At each site, each nudibranch species showed significant selectivity for two sponge species and consumed several others. The sponges consumed did not differ greatly among sites, even though sponge diversity differed among the three communities.

Our understading of nudibranch evolution is hampered by a lack of reliable phylogenetic characters. I describe several new characters based on the arrangement of calcareous spicules within slug bodies. These networks are reasonably consistent within known taxa, and may help resolve the affinities of several ambiguous taxa. With this information, I suggest that the species studied here may be basal to more specialized lineages, and that future phylogenetic studies of feeding specialization in this group could test which factors lead to narrow diet breadth.

## ACKNOWLEDGEMENTS

I am amazed at how little a thesis can be considered a solo enterprise. First of all, I would like to thank my committee members for their help over the years. Drs. Norm Stacey, Dave Hik and John Addicott greatly improved my scientific thinking as I brought in piece by piece of this project. I also thank Dr. Jim Nybakken for making the long trip to Edmonton to discuss nudibranchs and provide input during my defense.

Despite having graduated from University of Alberta, I actually spent most of my last six years at the marine station in Bamfield: a tiny town on the beautiful, wave-beaten west coast of Vancouver Island. I am continually amazed that, in this time of scarce resources, UA would happily provide for a graduate student who spent most of his time 1,400 km away, staring at the water. The Department of Biological Sciences provided crucial financial, logistic and intellectual support during that time.

I would never have gotten this far without the support of the station staff. Shane Servant and James Mortimor helping me with diving, Nathan Webb "co-ordinating" my research, and everyone who volunteered to dive or tidepool, often on short notice. My labmates have taught me a great deal as well, from driving boats to working Photoshop to basic worm identification. Jayson Gillespie, especially, should get an award for putting up with me longer than anyone else. I was also lucky to be at the marine station through two cohorts of graduate students, and much of this thesis was improved or conceived through discussions with them at happy hour or loitering behind the aquarium level. I sense I am not alone in this, and hereby nominate these two sites to share a Nobel Prize in marine ecology.

Many Bamfield residents helped expand my worldview and appreciation for life, but Nancy Christney and Brenda Hawkins deserve special mention. Their companionship formed many of my lasting memories, and their friendship kept me going when my tolerance for station life was exhausted. The station also gave me two extra mentors, Drs. Andy Spencer and John Holmes, who have continued to give advice and support over the years.

I've been extremely fortunate to teach during most of my tenure here. That experience has helped clarify not only what I needed to better investigate, but who I wanted to be. I'd like to thank Maggie Haag, Laura Verhegge and J.P. Danko for making that task so rewarding. I'd especially like to thank my students for their insights, curiousity and tolerance as I learned how to teach. I believe you all were cheated, as I learned far more from you than you ever did from me. But no, I'm not refunding your tuition.

As if this weren't enough, a few people made all the difference when the chips were down. Ron Koss taught me histology, how to deal with students, specimens and slugs, and mostly how to reconcile being salt-of-the-earth with being an academic. His enthusiasm for invertebrates, teaching and life in general is inspirational. David J. Arsenault was my "big-brother" in the lab, going to the field when the weather was rough, troubleshooting computers, helping me drain carborators, and very, *very* patiently explaining how ANOVAs worked. For the fifteenth time. To list all these two have done to help me would take longer than my thesis. Suffice to say I've felt priviledged for their company over the years and suspect I'll rarely meet such dear friends again.

My advisor, Dr. Rich Palmer, kept extremely high standards throughout this process, and has gone the extra mile(s) to help me achieve them. Moreover, he's been an extremely human advisor, showing me how to be a good scientist, to keep my integrity, and yet to not lose track of what is important in life. If our mutual induction of grey hairs has not been completely equal, I'm sure my own students will one day even the score.

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# CHAPTER 1

## GENERAL INTRODUCTION

Predation is an important determinant of community structure, but the actual effects of specific interactions on the community are determined both by which consumers are present and by what they find to be acceptable prey (Hay and Fenical 1988, Janzen 1988, Begon et al. 1990, Duffy and Hay 2001). Terrestrial plant communities are heavily influenced by insect grazers, many of which are quite specialized (Strong et al. 1984). Phytophagous insect diet breadth and composition are profoundly influenced by plant chemistry (Ehrlich and Raven 1964), raising interest in how secondary metabolites, and defense in general, affect insect host choice, and how these systems evolve (for review, see Feeny 1992). The identity and relative importance of these mechanisms is still the source of much contention (Strong 1988), but probably involves a balance between overcoming host defenses, physiological limitations of the consumer, and the need to obtain defense (Bernays and Graham 1988 and replies).

In contrast, specialized herbivores seem much less abundant and to have relatively less impact in marine systems (Lubchenco and Gaines 1981, Hay and Fenical 1988, Hay and Steinberg 1992, Paul et al. 2001). Although grazing pressure is intense – up to 100% of daily algal production removed on some tropical reefs (Carpenter 1986) – most marine grazers are large, fast-moving and generalized feeders (Duffy and Hay 2001). The smaller number of known marine specialists are usually "mesograzers": slow-moving, small animals that harbor more intimate associations with their prey to gain defense against larger generalist consumers (Hay and Fenical 1988, Duffy and Hay 2001, Paul et

al. 2001). Additionally, many benthic marine organisms seek refuges from predation by associating with well-defended species, even when the defended species is not used as prey (for review, see Duffy and Hay 2001, Stachowicz 2001). These data seemingly support the need for "enemy-free space" (*sensu* Bernays and Graham 1988) as a driving force behind feeding specialization. However, given that the prey of most grazers provide both food and shelter, the relative importance of these two factors is hard to distinguish (Stachowicz 2001) and tests of alternate systems are required. The prediction is that the most specialized feeders will be those that lack effective endogenous defenses, are smaller than their prey and do not often move far from it.

Opisthobranch molluscs include the most extensively investigated putative marine specialists (Hay and Fenical 1988), and nudibranchs in particular show great promise for testing hypotheses of feeding specialization based on insects and other arthropods (Karuso 1987). These slow-moving, shell-less, yet often flamboyantly colored marine gastropods are often associated with well-defended prey (Thompson 1976) and seem only rarely eaten by predators (Todd 1981). However, as grazing carnivores, nudibranchs present a very different system for investigation. The cryptobranchiate dorid nudibranchs are a monophyletic taxon (Schmekel 1985, Valdés and Gosliner 1999, Wägele and Willan 2000, Valdés and Gosliner 2001) feeding almost exclusively on sponges (McDonald and Nybakken 1997). Additionally, they are among the best-known examples of marine consumers using chemical defenses sequestered from their prey (Avila 1995), and this attribute is thought to have greatly influenced the evolution of the group (Faulkner and Ghiselin 1983, Cimino and Ghiselin 1999).

However, several mysteries remain. Other potential defenses and their roles relative to sequestered chemicals are poorly known (Todd 1981), despite some evidence that nudibranch defense is based on more than chemistry (Faulkner 1992). Likewise, the degree to which most species specialize on particular prey may be exaggerated. Although nudibranchs are reported to be feeding specialists (Thompson 1976, Cimino and Ghiselin 1999), one recent review suggests rather broad prey ranges for many common slugs (McDonald and Nybakken 1997). Because many feeding records are based solely on anecdotal reports that may not distinguish between use of a sponge as food versus substrate, it is unclear to what degree they reflect the real diet breadth of these species. Lastly, we are only beginning to properly resolve nudibranch phylogeny (Wägele and Willan 2000), so any current conclusions about the evolution of chemical defense or prey specificity for this group are tentative at best.

**Goals and organization of the thesis**

I sought to empirically test several aspects of both defense and feeding for this group, and to test a new phylogenetic character that may resolve relationships of several taxa for which the best feeding data are available. This thesis is divided into six chapters, including this introductory chapter (Chapter 1) and a general summary chapter (Chapter 6), plus two appendices. The four data chapters (2-5) and appendices are written for separate publication, and some repetition of information and arguments has been necessary to maintain this format.

**Chapter 2**. Lowered nutritional quality has been suspected as a defense for nudibranchs (Todd 1981) but has never been tested explicitly. Indeed, lowered nutritional content as a defense is often thought unavailable to mobile, solitary invertebrates (Cronin 2001). Defense by reduced nutritional value has two components: the organism must be less nutritious than other available prey, and predators must actually respond to this difference (Moran and Hamilton 1980). I surveyed members of all suborders of nudibranchs for proportional organic content – a proxy for calories provided – and, combining these observations with data from the literature, tested whether slugs are less nutritious than prosobranch snails. Finally, I tested whether differences in organic content comparable to those found between these gastropods can deter the sympatric generalist predator Cancer productus (Decapoda: Cancridae).

**Chapter 3**. Many dorid nudibranchs possess calcareous spicules as a high proportion of dry mass. Although often reported as a defense (Paine 1963, Todd 1981, Garcia et al. 1986), this role has never been directly tested (Foale and Willan 1987). For one common nudibranch (Cadlina luteomarginata), I surveyed the within-body pattern of spicule allocation to see whether it was consistent with a defensive role. I then assayed the deterrent effect of these spicules, alone and in combination with chemical defense, against several common generalist molluscivores. Finding evidence for only a minor deterrent effect, I then used histological techniques to assess spicules' potential structural role.

**Chapter 4**. Despite assertions that nudibranchs are specialized feeders, many common Northeastern Pacific slugs have a broad range of reported prey. The anecdotal reports upon which these broader prey ranges are based are supported by a few quantitative studies (Bloom 1981, Hellou et al. 1982, Thompson et al. 1982) that show reasonably broad diets at individual field sites. However, these studies lack data on relative sponge abundance in the field, so we cannot eliminate the possibility that slug diets are dictated simply by prey availability. I therefore surveyed several field sites in Barkely Sound, B.C. for sponge abundance and use by two species of nudibranch to test for prey selectivity. I then integrate these observations with those available from the literature to discuss potential differences in diets over the geographic ranges of these and other species.

**Chapter 5**. Any hypotheses of character evolution must be substantiated with robust phylogenies. However, because reasonable morphological characters for this group are difficult to identify (Wägele and Willan 2000), our understanding of nudibranch relationships is far from perfect (Karuso 1987). New techniques and characters have improved the situation, and led to several major rearrangements in the past few years (Valdés and Gosliner 1999, 2001). Nudibranch spicules are arranged in discrete networks that show some variation among taxa (Chapter 3, Garcia et al. 1986), and show some promise as a phylogenetic character (Wägele and Willan 2000). I investigated the form of these networks among several known monophyletic groups of Cryptobranchia, and attempted to determine usable characters and their polarity using common outgroup taxa. Lastly, I applied these characters to several species of uncertain

taxonomic affinity for which we have good data on both prey use and chemistry, to more confidently resolve their place within the Cryptobranchia.

**Notes on the conventions used.**

Many different nudibranch species appear in various chapters, and phylogenetic differences among them may later prove to alter the conclusions I have reached. Unfortunately, nudibranch taxonomy is somewhat unstable, with several competing systems (Karuso 1987), none of which have been universally supported. While this situation is gladly improving (see Chapter 5), studies using numerous species from multiple taxa can be confusing for the non-specialist. For clarity, I have included a table of the classification for all opisthobranchs used to provide data for this thesis (Table 1-1). This roughly follows Behrens' (1991) field guide to the fauna on this coast, incorporating the commonly accepted superfamily classifications for the dorids (e.g. Valdés and Gosliner 1999). This system suffers from the disadvantages mentioned above, and likely errs on splitting some natural groups or misplacing others, but it is currently the most accessible to those doing field biology.

How one uses statistics for hypothesis testing is likewise contentious (Johnson 1999). Unless otherwise noted, I have used the common convention of $p < 0.05$ as an arbitrary cutoff for statistical significance. While there are problems to this approach, I feel that it is a useful benchmark. However, I have tried to place more emphasis on the potential biological significance of observations.

**General significance**

The effects of predation on a community are moderated by two factors: 1) the degree to which consumer populations are controlled by abiotic factors and their own predators, pathogens, or competitors and 2) the range and identity of species they find acceptable as prey (Janzen 1988). Decades of research on this second topic have focused on insects and plants, because "the overwhelming majority of all species interactions occur between herbivorous insects and plants" (Strong 1988). Although such work has helped clarify the factors that determine diet breadth and composition in this context, tests in other systems are required to separate general principles from taxonomic biases. By using marine, molluscan grazing carnivores, I hope to provide a useful foil to this previous research in terrestrial systems.

**Table 1-1.** Classification of species used in the thesis. Classification modified from Behrens (1991).

**ORDER NOTASPIDEA**

Family Pleurobranchidae

Berthella californica (Dall, 1900)

**ORDER NUDIBRANCHIA**

SUBORDER DORIDACEA

Family Doridoxoidea

Bathydoris aioca Marcus and Marcus 1962

Superfamily Phanerobranchia

Family Goniodorididae

Hopkinsia rosacea MacFarland, 1905

Family Onchidorididae

Acanthodoris hudsoni MacFarland, 1905

Acanthodoris nanaimoensis O'Donoghue, 1921

Acanthodoris rhodoceras Cockerell and Eliot, 1905

Family Notodorididae

Aegires albopunctatus MacFarland, 1905

Family Polyceratidae

Polycera atra MacFarland, 1905

Triopha maculata MacFarland, 1905

Superfamily Cryptobranchia

Family Hexabranchidae

Hexabranchus sanguineus (Rupell and Leuckart, 1828)

Family Chromodorididae

Cadlina luteomarginata MacFarland, 1966

Family Aldisidae

Aldisa cooperi Robilliard and Baba, 1972

Aldisa sanguinea (Cooper, 1863)

Family Doridiidae

Atagema alba (O'Donoghue, 1927)

Family Asteronotidae

Sclerodoris coriacea Elliot 1904

Family Archidorididae

Archidoris montereyensis (Cooper, 1863)

Family Discodorididae

Anisodoris nobilis (MacFarland, 1905)

Diaulula sandiegensis (Cooper, 1963)

Family Dendrodorididae

Dendrodoris albobrunnea Allan, 1933

Doriopsilla albopunctata (Cooper, 1863)

Doriopsilla areolata Bergh, 1880

Family Phyllidiidae

Phyllidia varicosa Lamarck, 1801

Phyllidiopsis cardinalis Bergh, 1875

SUBORDER DENDRONOTACEA

Family Dendronotidae

Dendronotus iris (Cooper, 1863)

SUBORDER AEOLIDIACEA

Family Flabellinidae

Flabellina iodinea (Cooper, 1862)

Family Facelinidae

Hermissenda crassicornis (Eschscholtz, 1831)

SUBORDER ARMINACEA

Family Dironidae

Dirona albolineata MacFarland in Cokerell and Elliot, 1905

Dirona picta Hurst, 1966

Family Zephyrinidae

Janolus fuscus (O'Donoghue, 1924)

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# CHAPTER 2

## LOWERED NUTRITIONAL QUALITY SUPPLEMENTS NUDIBRANCH CHEMICAL DEFENSE[1]

### Introduction

Organisms unable to run or hide once detected often exhibit other ways to deter consumption by predators (Lima and Dill 1990). Physical defenses such as the shells of snails or the calcified thalli of seaweeds explain some species' resilience (Duffy and Hay 2001). Species lacking physical defenses often possess deterrent chemicals, often with novel structures (Paul 1992, Cimino et al. 1999). Physical and chemical defenses have historically been the focus of most studies in the marine environment (Duffy and Paul 1992, and references therein), yet other potential defenses exist (Chanas and Pawlik 1995). One strategy is simply to not be worth eating (Cronin 2001); predators seek to maximize energy gained and minimize the cost of consuming prey (Hughes 1980), so organisms that present little net nutrition may enhance the effectiveness of their other defenses.

Such increased defense via reduced nutritional quality has been demonstrated for many sessile organisms that suffer partial predation, but rarely has been shown in solitary, mobile animals. Changes in palatability based on both secondary chemistry and nutritional value have been increasingly considered for prey as diverse as terrestrial angiosperms (Potter and Kimmerer 1986, Schmitz et al. 1992, Agrell et al. 2000),

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seaweeds (Johnson and Mann 1986, Duffy and Paul 1992, Hay et al. 1994), sponges (Pennings et al. 1994), and gelatinous holoplankton (Bullard and Hay 2002). However, predator preferences do not always correlate with nutritional quality (Chanas and Pawlik 1995, Stachowicz and Lindquist 2000) and reduced nutritional value can lead to increased compensatory feeding under some conditions (Cruz-Rivera and Hay 2000). Regardless, nutritional quality as a defense is thought unavailable to mobile invertebrates, because their tissue is more nutritious and reduction of nutritional content limits material available for growth and maintenance functions (Cronin 2001). Plants and colonial invertebrates can sacrifice less-valuable units (e.g., leaves, zooids, etc.) (Cronin 2001), while gelatinous animals such as cnidarians are already constrained to two tissue layers (Brusca and Brusca 1990). Termites are also defended by nutritional quality as well as chemical and physical defenses, but each colony usually only suffers partial predation (Redford 1984, 1985). Social insects share with colonial invertebrates several characteristics that may favor polymorphism among individual units (Harvell 1994), possibly mitigating the cost of maintaining units at lower nutritional value. Flesh of other animals does vary somewhat in nutritional content (Vinogradov 1953, Redford and Dorea 1984). Can such variation also defend mobile invertebrates?

Nudibranchs (Opisthobranchia: Gastropoda) are one taxon for which chemical defenses are often tested with little other context. Despite often being flamboyantly colored, slow-moving, and unconcealed on contrasting substrate, these sea slugs have few documented predators (Thompson 1976). In contrast to prosobranch gastropods– shelled marine snails– opisthobranch gastropods such as nudibranchs, sacoglossans and cephalaspideans have reduced or absent shells (Thompson 1976) and must rely on other

defenses. Many nudibranchs possess novel secondary chemicals and, although not all have been specifically tested, many deter predators (Cimino et al. 1999). However, sea slugs possibly require other lines of defense to survive: nudibranch secondary chemicals may not be effective against all predators (Faulkner 1992), and are often sequestered from particular prey (Avila 1995) that may not always be available. While some nudibranchs also contain sulfuric acid or diet-derived nematocysts (Thompson 1976), many species lack such obvious deterrents. Defense by low nutritional quality has been suspected for nudibranchs that contain calcareous spicules (Todd 1981), but has not been rigorously investigated.

I report here that both spiculated and unspiculated nudibranchs contain fewer calories per unit body mass than sympatric, prosobranch gastropods. Nudibranchs also invest organic material differentially by tissue, with exposed tissue the least nutritious, the best defended, and often easily replaceable. Changes in nutritional quality of this magnitude can reduce feeding by generalist predators in no-choice assays. Finally, I argue that, given evidence from antipredatory assays and foraging theory, differences in nutritional quality could supplement other defenses of mobile animals, and this should be considered in studies of chemical defense as it would be for organisms suffering partial predation.

## Methods

### Whole body

One to three individuals each from several gastropod species (five nudibranchs, eight prosobranchs) were collected subtidally from Barkley Sound, B.C. Shells and

opercula were removed, and individuals damp-weighed. Ash-free dry mass was determined via standard procedures (Paine 1964) to calculate organic content per unit wet mass. These data were combined with other published values where the same author provided both ash and water content for a given species (Paine 1963, 1964, Menge 1972); each species mean from the literature was counted as a single observation in the analysis. Values for each species are given in Appendix 2-A. I assigned nudibranch species into subgroups based on the presence or absence of calcareous spicules in their tissues. Comparisons among subgroups were made by Analysis of Variance (ANOVA) on arcsine-transformed proportions (Sokal and Rohlf 1981). Two separate analyses were performed: a) a two factor design with individuals as replicates within species, nested within groups, and b) a one factor design using species means as replicates within groups. As the statistical differences among groups did not differ between the two analyses, only the simpler analysis is presented below.

Tissue

One to three individuals each from several gastropod species (seven nudibranchs, three prosobranchs) were collected subtidally from Barkley Sound, B.C. Animals were dissected into mantle margins (including cerata), central mantle, foot, and viscera, then individually damp-dried and weighed. Organic content was determined as for whole bodies. Differences among groups and body regions were compared using a three factor, mixed-model ANOVA on arcsine-transformed proportions (Sokal and Rohlf 1981) with individual observations as replicates. Normally, in a two-way comparison between Region and Group, both would use the residual MS as denominator (Sokal and Rohlf

1981). However, Species [G] is nested within Groups; therefore this F-ratio uses the Species [G] MS as denominator (Table 2-2b), corrected for unequal sample size as 0.9771 * Species [G] + 0.0229 * Residual (JMP Statistical Package, version 4). Values for each species are listed in Appendix 2-B.

Bioassays

To test whether the observed differences in organic content affected palatability to predators either alone or in combination with chemical defense, I assayed artificial food of manipulated organic and chemical content against a generalist predator known to consume gastropods. Cancer productus, a common shallow-water molluscivorous crab species (Orensanz and Gallucci 1988), were collected by baited trap from Bamfield Inlet, B.C., individually maintained in flowing natural seawater at the Bamfield Marine Station, and fed squid mantle (ca. 5 $cm^2$ pieces) every other day. This regime was meant to ensure that crabs were trained to accept food in the laboratory, and would be hungry, but not starving, for the assays.

Four types of artificial food were made using a combination of freeze-dried, powdered squid mantle and sodium alginate, modified from Lindquist and Hay (1996). This recipe allowed consistent control of food quality, although ash composition was slightly higher than most natural foods (Table 2-1). Food was made to either low or high organic content (12% or 22% of wet mass, respectively) by adjusting the ratio of squid powder to water. The low quality food approximated the median organic content of spiculated nudibranch whole bodies (Appendix 2-A) and foot tissue of Archidoris montereyensis (Appendix 2-B). Because there was an upper limit at which addition of

squid powder prevented artificial food from solidifying adequately, the high quality food contained slightly less organic material per unit wet weight than average snail bodies (Fig. 2-1) or tissues (Fig. 2-2). While the organic contents of these artificial food types are realistic, they most closely approximate the minimum difference between snail and nudibranch flesh. Therefore, this experiment may slightly underestimate the average difference in predator response that would be seen in the field.

During preparation, dry components were treated with either methanol alone or whole methanolic extracts of Archidoris montereyensis (Nudibranchia: Doridacea) at concentrations matching those of nudibranch tissue, and the solvent was allowed to evaporate before adding water. These extracts contain a terpenoic acid glyceride and a glyceryl ether previously shown to deter feeding by tidepool sculpins (Oligocottus maculosus) (Gustafson and Andersen 1985).

Food pellets were offered to crabs in no-choice assays. Crabs received one treatment per day in haphazard order, and each crab then received all treatments again, in a different order. For each test, the pellet was offered to the crab until it was ignored for two minutes, at which point all remaining food was siphoned from the tank and dried at 60°C. The proportion of each pellet consumed was calculated as change in dry weight, to remove potential confounding effects of water absorption by the food. The remaining dry weight was divided by the estimated initial dry weight (IDW), which was calculated from initial wet weight (IWW) using regressions based on control pellets (High quality: $n = 6$, $IDW = (0.1452)(IWW)+0.27$, $r^2 = 0.815$, $p = 0.014$; Low quality: $n = 6$, $IDW = (0.157)(IWW) + 0.023$, $r^2 = 0.950$, $p = 0.001$). In total, four treatments were twice offered to each of fourteen crabs.

To test for losses independent of crab handling, a second group of control pellets, either whole or shredded with forceps, was left in arenas without crabs for five minutes and then recovered as above. Differences among treatments were tested using ANOVA on arcsine-transformed proportions. Shredding reduced recovery by approximately 10% ($df = 1$, $MS = 0.080$, $F = 35.2$, $p = 0.003$). Neither nutritional quality ($df = 1$, $MS = 0.000$; $F = 0.0$, $p = 0.891$) nor interaction between quality and degree of manipulation ($df = 1$, $MS = 0.004$, $F = 1.7$, $p = 0.231$) affected recovery. Thus, recovery of material should have been equal among treatments, unless crabs differentially consumed or attacked (i.e. shredded) pellets.

Potential effects of extracts, organic content and individual crab were analyzed by a randomized block factorial ANOVA (Sokal and Rohlf 1981). One crab consuming less than 25% of the high organic content food without extracts in both trials was excluded from the analysis as lacking sufficient motivation to feed. Because data were proportions, all were arcsine-transformed before analysis to promote normality. Homogeneity of variances for individual crabs and treatments were assessed by a two-factor ANOVA on absolute differences between arcsine-transformed feeding proportions of the two trials. Because there was effectively no error term over which to test effects in this heterogeneity analysis, I assumed the Treatment x Individual interaction was not significant and used this mean square as the denominator for the other F-tests (Sokal and Rohlf 1981). Other potential confounding effects included learning or satiation of individual crabs over the experiment; both would likely reduce feeding as the assay proceeded. Therefore, I used a pairwise t-test (Sokal and Rohlf 1981) to assess whether differences between the first and second trials differed significantly from zero.

Some authors disagree on the proper methods to test for synergism among defenses. ANOVA tests for deviations from simple additive effects, so non-significant interactions likely preclude the need for further testing (Hay 1996). However, synergistic response to defenses may be mulitplicative rather than linear (Pennings 1996). As an added precaution, I also asked whether nutritional quality disproportionately changed the effect of chemical extracts by calculating K-factors of extract for both high and low quality pellets (e.g., Addicott and Bao 1999) as $\log_{10}$(control) - $\log_{10}$(extract treated), using mean feeding values for each crab as replicates. Because these original proportions included some zero or negative values, all were transformed to range between 0.001 and 1 by adding the greatest outlier to both numerator and denominator ($x_1 = (x + 0.056) / 1.056$). Pairwise differences among K-factors were tested using a student's t-test (Sokal and Rohlf 1981).

This repeated, no-choice, ANOVA-based design is unusual compared to previous papers (Duffy and Paul 1992, Hay et al. 1994), but has several advantages. First, an ANOVA design, rather than qualitative comparison of independent trials, allows more rigorous, direct testing of both the effect of nutritional quality and its interaction with chemical defense. Second, offering one piece of food at a time tested deterrence, rather than relative preference, for each treatment; it also offset the lack of independence among treatments inherent in cafeteria experiments (Peterson and Renaud 1989). Third, repeated observations on each crab allowed better estimation of each crab's response to the various treatments, and therefore: a) whether individuals respond differently to either type of defense, and b) whether crab response changed over the course of the experiment, due to learning or satiation.

**Results**

Whole body

All nudibranchs contained significantly less digestible organic material (ash-free dry mass) to wet mass than prosobranch snails ($P < 0.0001$, ANOVA; all pairwise differences $p < 0.005$, Fisher's PLSD). Specifically, both groups of nudibranchs contained less than 12% organic material by weight, or less than half that of prosobranch snails (Fig. 2-1A, Organic). Although individual species within groups also differed significantly (nested ANOVA, $p < 0.002$), this did not affect the significance of among-group comparisons.

The material responsible for diluting organic content varied between groups of nudibranchs. Slugs with spicules contained strikingly higher amounts of ash per unit wet weight, while nudibranchs lacking spicules and prosobranchs both contained approximately 3% ash, the expected amount from tissue salts (Fig. 2-1A, Ash). Regardless, all nudibranchs had higher water content than prosobranchs, although slugs with spicules had lower water content than slugs lacking them (Fig. 2-1B).

Tissue

Organic content was always lower in the tissues of nudibranchs than in those of prosobranchs (Fig. 2-2). The pattern of organic investment in different tissues varied significantly among gastropod groups (Table 2-2c), as nudibranchs exhibited proportionally less organic material in exterior body regions than in the viscera, unlike

prosobranch snails where proportional organic content did not vary among body regions. Differences between groups of nudibranchs were not statistically significant.

Bioassays

Experimentally lowered organic content reduced feeding by Cancer productus. In no-choice assays, hungry crabs consumed less of artificial food pellets with the low organic content of nudibranchs than of pellets with the higher organic content of snails (Fig. 2-3; Table 2-3b). The addition of extracts also significantly reduced feeding (Fig. 2-3; Table 2-3a). Distribution of residuals did not significantly differ from normality (Shapiro-Wilks test. $W = 0.979$, $p = 0.492$), and no treatment or crab contributed disproportionately to the overall variance (ANOVA. Treatment: $df = 3$, $MS = 0.006$, $F = 0.135$, $p = 0.939$; Individual: $df = 12$, $MS = 0.38$, $F = 0.873$, $p = 0.581$). Feeding rates were not significantly lower in the second trials (one tailed, paired T-test. $N = 45$, mean = 0.023, S.E.= 0.51, $p = 0.325$), indicating crab feeding was not unduly affected by learning or satiation.

The effect of nudibranch extract did not depend on the level of nutritional quality, either detected as an interaction (Table 2-3c) or by comparison of proportional changes (K-factors; one-tailed paired T-test: $n = 12$, mean, -0.144; S.E. = 0.129, $p = 0.143$). Response of individual crabs varied (Table 2-3, d) due to differences in mean feeding rates. The Individuals term did not interact significantly with either treatment. However, the interaction between Individuals and the combination of Organic content and Extracts bordered on statistical significance ($p = 0.055$; Table 2-3g).

**Discussion**

Nudibranchs contained less organic matter per unit wet mass than prosobranch snails (Fig. 2-1A, Organic), and this may affect their value as food to potential predators. Opisthobranchs in general may have reduced organic content, as reported water contents of some other nudibranchs (Vinogradov 1953), sacoglossans (Pennings and Paul 1993) and cephalaspideans (Paine 1963) are also higher than those reported for snails and many other marine molluscs (Vinogradov 1953, Menge 1972). Because each unit of nudibranch ash-free dry mass contains the same or fewer calories than those of prosobranchs or other marine animals (Paine 1964, Todd 1979), lower proportional organic content means lower proportional energy content. Consumers often forage to maximize net energy return per unit handling time of prey (Hughes 1980). Handling time and energy expenditure are somewhat greater for snail prey, which have shells that must be removed or punctured for consumption (Norton 1988). However, some species and sizes of shelled prey require minimal time and energy to consume (Creswell and McLay 1990), so direct comparisons are required to assess the true difference in net value of snails versus slugs.

Both extra water and ash contributed to lowered organic content of nudibranchs. Slugs contained more water per unit mass than snails, although this difference was less for slugs that contained spicules (Fig. 2-1B). This extra water might be partially a consequence of the expanded hemocoelic space in these animals (Uyeno 1999); however, dissected nudibranch tissues also had lower organic content than those of snails (Fig. 2-2), suggesting some extra water lies in the tissues themselves. The remaining water is perhaps partially contained in the large fluid-filled vacuoles for mucus and defensive

glands in outer tissues (Hyman 1967). Some nudibranchs– mostly cryptobranchiate dorids– contain spicules of calcium carbonate (Thompson 1976) and therefore more than double the ash per unit mass of snails or other slugs (Fig. 2-1A, Ash). Aside from diluting tissue value, these spicules could also defend nudibranchs by mechanically abrading consumer guts or inhibiting acidic digestion (Hay et al. 1994), or they could serve a non-defensive, structural role (Koehl 1982). However, regardless of "filler" material used, both types of nudibranchs exhibit a similarly low proportion of organic material per unit volume. Just as chemical defense may have led to the loss of shells in these gastropods (Faulkner and Ghiselin 1983), adoption of a slug-like form with a large hemocoel (Uyeno 1999) may be a preadaptation to lowered nutritional content.

Reduced food value through increased water or ash content may be widespread among some animal taxa. Those possessing extensive fluid or gelatinous body matrices could quite easily have reduced nutrient content (Chanas and Pawlik 1995, Cronin 2001) and for gelatinous holoplankton, reduced protein content almost certainly contributes to predator deterrence (Bullard and Hay 2002). Other terrestrial and marine invertebrates vary in organic content per unit wet mass (Vinogradov 1953, Redford 1984, 1985), and both deep-sea crustaceans and fish have reduced nutritional value (Nybakken 1997, and references therein). For aquatic and marine invertebrates, increased water content could entail little cost: water is readily available, can be obtained through osmosis or exterior body openings, and would not necessarily increase metabolic demands for maintenance or movement through aqueous media. Even the 'cost' of lowered nutrient concentrations in the tissues (Moran and Hamilton 1980) might be offset if excess water was contained in vacuoles. As well as directly deterring consumption by predators, increased water

content would permit increased size for a given organic investment, and increased size alone may deter some predators (Paine 1976). The limiting factor may be the capacity to store nutrients for other functions, such as maintenance and reproduction (Cronin 2001).

The distribution of organic material within nudibranch bodies differed from prosobranch snails, and might further reduce desirability as prey. Nudibranchs contained proportionally less organic material per unit mass in their outer tissues than in their viscera; whereas prosobranchs exhibited no difference (Fig. 2-2). A similar pattern has been reported previously for the nudibranch Archidoris pseudoargus (McCance and Masters 1937). Reduced allocation of organic material to exterior body regions such as cerata or mantle margins decreases the apparent nutritional content– predators taste these outer areas first– while maintaining a somewhat higher nutrient store in the viscera. Additionally, mantle margins and cerata also contain higher levels of chemical defense (Kubanek et al. 2000) or sequestered nematocysts (Thompson 1976), and can often be removed or autotomized without apparent harm to the nudibranch (Hyman 1967). Therefore, the areas of slug bodies most vulnerable to attack are the best defended, the least valuable to potential predators, and the least costly for nudibranchs to replace.

Reduced nutritional value only offers a selective advantage to non-modular animals if potential consumers detect and reject prey on this basis (Moran and Hamilton 1980). C. productus consumed approximately 19% less of food with reduced organic content than of food which matched the organic content of snails (Table 2-3b; Fig. 2-3). For comparison, addition of chemical extracts reduced feeding by approximately 50% (Table 2-3a; Fig. 2-3). Responses to nutritional quality and chemical defense did not differ significantly among individual crabs (Table 2-3e, f), although mean feeding rate

differed among individuals (Table 2-3d). No crab or treatment contributed significantly to the overall variance, and feeding rate differences between replicate trials were not significantly different from zero. Therefore, these results are unlikely due to learning, satiation or sporadic feeding of crabs in certain trials. Although the lower quality food was consumed consistently less than the high quality food, crabs were nonetheless fed a constant, higher quality diet before the assays, and their decisions were possibly influenced by a perceived "average prey" of this environment (Krebs and Davies 1997). Because gut volume and time both limit how much consumers can eat (Belovsky and Schmitz 1994), they may reject seemingly poor quality prey for the chance to ingest higher quality prey later. Such differences in preference due to water content are seen in other systems, e.g. changes in dry mass/wet mass can change the effects of plant chemical defense on snowshoe hare foraging (Schmitz et al. 1992).

Nutritional content does not always act synergistically with other defenses. I found no interaction between the effects of nutritional quality and chemical defense (Table 2-3c; K-factor analysis), suggesting that nutritional quality alone can influence acceptability. Similarly, in field experiments with herbivorous reef fish, differences in protein content affected prey choice as much as the presence of deterrent chemicals, and the effects of nutritional quality were independent of those of chemical defense (Duffy and Paul 1992). However, another study on seaweeds found little deterrent effect due to organic content alone, but that the effect of chemical defense was stronger when organic content was reduced (Hay et al. 1994). These studies tested different consumers, types of nutritional quality and secondary compounds, so the reason for this discrepancy is unclear. Individual crabs may respond differently to the combination of nutritional

quality and chemical defense (Table 2- 3, g), so the actual effect may depend to some degree on individual hunger, motivation or previous experience (Lindroth 1988, Distel and Provenza 1991).

Decreased consumption of the magnitude caused by lowered caloric content could be beneficial, even without being completely deterrent. Predators search for prey yielding higher energy per unit handling time (Hughes 1980, Krebs and Davies 1997), so consumption of lower quality prey is subsequently reduced. Because predators can learn to judge prey quality quickly and without necessarily inflicting damage (Hughes and Seed 1981), attacks may not always be lethal. Indeed, nudibranchs (Appendix II) and other soft-bodied solitary organisms may survive such sampling, and another chance to reproduce is certainly advantageous compared to immediate death. Because potential prey species often abound in the natural environment and predators will likely focus on the most profitable ones, any prey trait that leads to even minor changes in acceptability may disproportionately increase survival. As in the old adage: one needn't be able to outrun the bear if one can outrun a companion.

Although differences in nutritional content within the range naturally occurring among organisms can significantly affect consumer foraging (Hughes 1980, Krebs and Davies 1997), current evidence that it actually protects species in natural settings is equivocal. Nutritional quality influences fish preference for seaweeds to a similar degree as chemical defense (Duffy and Paul 1992) and, combined with nematocysts, accounted for approximately 90% of the deterrence of gelatinous holoplankton to fish (Bullard and Hay in review). However, consumer preference is often not correlated with nutritional content alone. Lipid content was the only one of several measures of quality even

slightly correlated with preferred sponge prey of fish (Chanas and Pawlik 1995) and nutritional content (measured as ash-free dry mass or protein content) made no apparent contribution to hydroid defense (Stachowicz and Lindquist 2000). Likewise, sea star choice of sponge prey was not apparently influenced by nutritional content (Waddell and Pawlik 2000). Effects of prey nutritional quality may be swamped by confounding factors in correlational studies, as consumers balance the need for nutrients against the need to avoid both avoid prey defenses and their own predators and to optimize other conditions (Lindroth 1988, Belovsky and Schmitz 1994).

Finding the conditions under which particular defenses are effective alone or in combination is an important goal. The efficacy of any defense is context-dependent (Belovsky and Schmitz 1994), so we need to better evaluate how nutritional quality (gain for predators) and defense (cost to predators) interact to protect potential prey. One-factor studies of chemical defense are of decreasing utility. For instance, compounds showing "no activity" using high-quality experimental food such as shrimp pellets (Hellou et al. 1982, Thompson et al. 1982) may significantly reduce predation when tested at an organism's natural food value, as seen with some sponge compounds (Pennings et al. 1994). Much stronger arguments about a compound's deterrency can been made in cases where nutritional quality is also considered (Chanas and Pawlik 1995, Bolser and Hay 1996). While this approach has become increasingly common for studies on plants and modular animals, it should be extended to other animals such as mobile invertebrates: energy gained is an important consideration for predators, whether their prey are sessile or mobile, eaten whole or a few bites at a time. Determining why and

when particular features deter predation will help us better understand the selective pressures that have formed current ecological communities.

**Table 2-1.** Artificial foods used in bioassays. A. Recipes used. B. Percent composition (mean ± standard error; n = 4). Each batch made one pellet of approximately 2 g.

A.

| Ingredient | Low-Quality Food (Nudibranch) | High-Quality Food (Prosobranch) |
|---|---|---|
| Squid powder | 0.3 g | 0.6 g |
| Water | 1.7 g | 1.4 g |
| Sodium Alginate | 0.4 g | 0.4 g |

B.

| Component | Low-Quality (Nudibranch) | High-Quality (Prosobranch) |
|---|---|---|
| Organic | 12.1 % (0.4) | 21.4 % (0.7) |
| Ash | 4.7 % (0.5) | 5.9 % (0.7) |
| Water | 83.2 % (0.7) | 72.7 % (1.0) |

**Table 2-2.** Results of ANOVA for organic content by body region for three gastropod groups. df, degrees of freedom; MS, mean squares; F, F ratio; P, exact probability. Sources of variation and levels were: Region (fixed; Foot, Mantle Margin, Central Mantle and Viscera), Group of gastropod (fixed; Prosobranch, Spiculated Nudibranch, Unspiculated Nudibranch), and Species within each group (random). Each species measurement was based on 2 - 3 individuals. See Figure 2.

| Source | df | MS | F | p | Denominator MS for F ratio |
|---|---|---|---|---|---|
| a) Region | 3 | 0.0367 | 22.4 | < 0.001 | Residual |
| b) Group | 2 | 0.3075 | 11.1 | 0.007 | Species [G]† |
| c) R x G | 6 | 0.0060 | 3.6 | 0.003 | Residual |
| d) Species [G] | 7 | 0.0282 | 17.2 | < 0.001 | Residual |
| Residual | 80 | 0.0016 | | | |

† Corrected for unequal sample sizes. See Methods for details.

**Table 2-3.** Results of ANOVA for bioassays on Cancer productus consumption of artificial food pellets. Sources of variation and levels were: Extracts of nudibranch secondary chemicals (fixed; Present or Absent), Organic content (fixed; High or Low), and Individual crab (random; n = 12). In total, four treatments were independently offered twice each to fourteen crabs. Abbreviations as in Table 2- 2.

| Source of variation | df | MS | F | P | Denominator MS for F ratio |
|---|---|---|---|---|---|
| a) Extract | 1 | 9.774 | 102.0 | < 0.001 | E x I |
| b) Organic content | 1 | 1.788 | 54.3 | < 0.001 | O x I |
| c) E x O | 1 | 0.009 | 0.0 | 0.787 | E x O x I |
| d) Individual crab | 11 | 0.509 | 8.8 | < 0.001 | Residual |
| e) E x I | 11 | 0.096 | 1.2 | 0.113 | Residual |
| f) O x I | 11 | 0.033 | 0.6 | 0.842 | Residual |
| g) E x O x I | 11 | 0.133 | 2.0 | 0.055 | Residual |
| Residual | 45 | 0.058 | | | |

**Figure 2-1.** Organic, ash and water content components of three gastropod groups, expressed as percent of wet mass (mean ± standard error). A) Groups of gastropods differ in proportional organic content as measured by ash-free dry mass ($p < 0.0001$, ANOVA; all pairwise differences $p < 0.005$, Fisher's PLSD). Spiculated nudibranchs contain approximately twice the ash of other groups. B) Nudibranchs have higher water contents than prosobranchs, although this difference is greater for nudibranchs lacking spicules. N = number of species in each group.

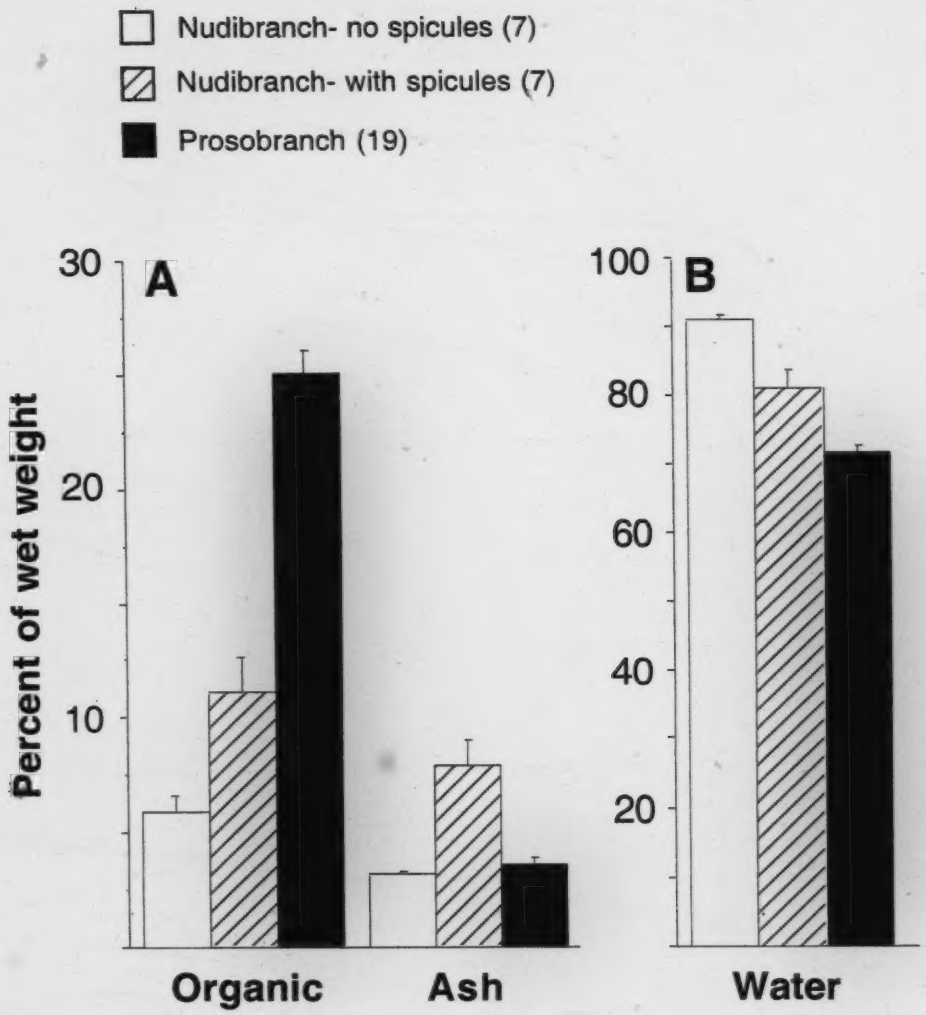

Nudibranch- no spicules (7)
Nudibranch- with spicules (7)
Prosobranch (19)
Percent of wet weight
30
20
10
A
100
80
60
40
20
B
Organic
Ash
Water

**Figure 2-2.** Organic content by body region for three gastropod groups, expressed as percent of wet mass (mean ± standard error). Animals were dissected into Foot, Mantle margin (Margin), Central mantle (Center), and Viscera. Nudibranchs contained less organic matter than prosobranchs in all tissues, and possessed relatively less in external body regions. Sample sizes for each group were (total individuals / species): Prosobranchs (7 / 3), Spiculated Nudibranchs (10 / 4), and Unspiculated Nudibranchs (8 / 3). See Table 2-2 for statistical analysis.

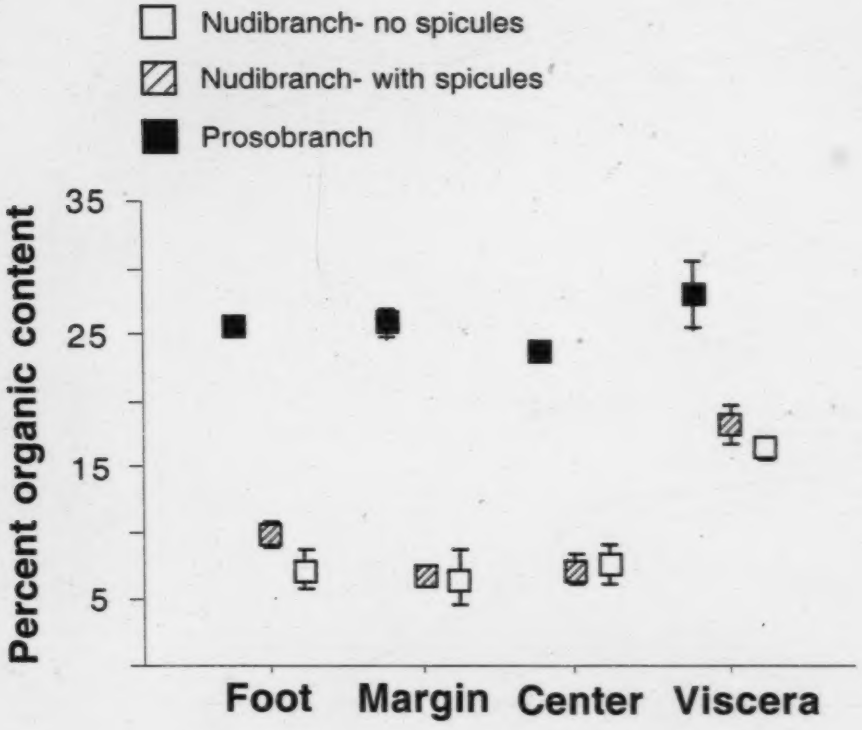
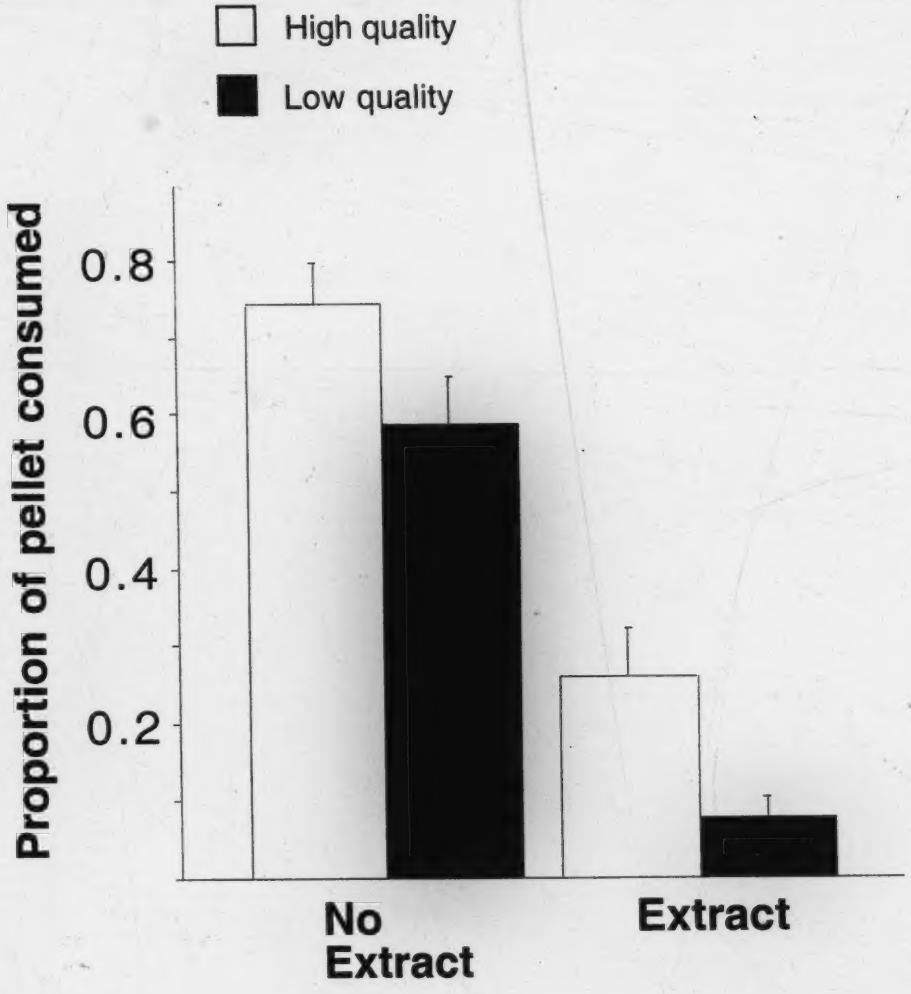

Nudibranch- no spicules
Nudibranch- with spicules
Prosobranch
Percent organic content
35
25
15
5
Foot
Margin
Center
Viscera

**Figure 2-3.** Results of no-choice laboratory assays testing the relative effect of food quality and chemical extracts on feeding by Cancer productus (Crustacea: Decapoda). Food pellets of either High or Low quality (see Table 2-1) were made either with nudibranch whole-body extracts (Extract) or solvent alone (No extract). Pellets were presented in haphazard order as individual pellets; data presented are proportion of pellet consumed in for each treatment (mean ± standard error). See Table 2-3 for statistical analysis.


High quality
Low quality
Proportion of pellet consumed
0.8
0.6
0.4
0.2
No Extract
Extract

**Appendix 2-A.** Percent Body Composition of Various Gastropods.

S = Source of data. a = Paine, 1963; b = Paine, 1964; c = Menge, 1972; no label = current study. Org = Organic content. SE = Standard Error, given when known. * = weighted average of original and published values (preceeding two rows), counting the literature report as one observation. Sources and sample sizes are listed for water content first and then ash, if these sources differed.

| Species | S | n | Water | SE | Ash | SE | Org |
|---|---|---|---|---|---|---|---|
| **Prosobranchs** | | | | | | | |
| ***Acmaea digitalis*** | c | 12 | 77.10 | | 2.86 | | 20.04 |
| ***Acmaea mitra*** | | 3 | 78.12 | 0.95 | 2.95 | 0.10 | 18.94 |
| ***Acmaea paradigitalis*** | c | 6 | 75.10 | | 2.56 | | 22.34 |
| ***Acmaea pelta*** | c | 4 | 76.40 | | 2.71 | | 20.89 |
| ***Acmaea scutum*** | c | 11 | 78.50 | | 2.45 | | 19.05 |
| ***Calliostoma canaliculata*** | | 1 | 73.30 | | 2.86 | | 23.84 |
| *Calliostoma ligatum* | c | 4 | 69.96 | | 4.66 | | 25.37 |
| *Calliostoma ligatum* | | 3 | 68.88 | 3.44 | 4.90 | 1.78 | 26.22 |
| ***Calliostoma ligatum**** | | 4 | 69.96 | 2.66 | 4.66 | 1.28 | 25.37 |
| ***Ceratostoma foliatum*** | | 2 | 69.96 | 3.81 | 3.25 | 0.37 | 26.79 |
| ***Lacuna sp.*** | c | 2 | 76.80 | | 2.71 | | 20.49 |
| ***Lirabuccinum (Searlesia) dira*** | c | 12 | 70.00 | | 3.00 | | 27.00 |
| ***Lithopoma gibberosum*** | | 2 | 72.26 | 0.94 | 3.55 | 0.05 | 24.19 |
| ***Littorina scutulata*** | c | 4 | 62.30 | | 4.60 | | 33.10 |
| ***Littorina sitkana*** | c | 8 | 68.10 | | 3.70 | | 28.20 |
| ***Margarites sp?*** | c | 5 | 60.80 | | 6.51 | | 32.69 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| ***Nucella (Thais) canaliculata*** | c | 11 | 73.20 | | 3.06 | | 23.74 |
| ***Nucella (Thais) emarginata*** | c | 8 | 70.80 | | 2.74 | | 26.46 |
| *Nucella (Thais) lamellosa* | c | 8 | 73.50 | | 3.23 | | 23.27 |
| *Nucella (Thais) lamellosa* | | 3 | 69.54 | 0.85 | 2.86 | 0.06 | 27.60 |
| ***Nucella (Thais) lamellosa**** | | 4 | 70.53 | 1.16 | 2.95 | 0.10 | 26.52 |
| ***Oceanebra lurida*** | | 2 | 58.86 | 0.66 | 6.83 | 1.05 | 34.31 |
| ***Tegula brunnea*** | | 3 | 72.24 | 1.30 | 3.58 | 0.25 | 24.18 |
| **Unspiculated nudibranchs** | | | | | | | |
| ***Dirona albolineata*** | | 1 | 94.06 | | 3.15 | | 2.79 |
| ***Dirona picta*** | a,b | 3,4 | 92.10 | | 3.24 | | 4.66 |
| ***Flabellina iodinea*** | a,b | 8,7 | 90.30 | | 2.91 | | 6.79 |
| ***Hermissenda crassicornis*** | a,b | 15,9 | 90.30 | | 2.72 | | 6.98 |
| ***Janolus fuscus*** | | 1 | 93.57 | | 2.98 | | 3.45 |
| ***Polycera atra*** | a,b | 8,6 | 89.80 | | 2.86 | | 7.34 |
| ***Triopha maculata*** | a,b | 16,19 | 89.00 | | 2.97 | | 8.03 |
| **Spiculated nudibranchs** | | | | | | | |
| ***Acanthodoris rhodoceras*** | a,b | 3,4 | 90.50 | | 3.90 | | 5.61 |
| ***Aegires albopunctatus*** | a,b | 3,3 | 71.00 | | 12.47 | | 16.53 |
| ***Aldisa cooperi*** | | 2 | 79.52 | 1.12 | 7.83 | 0.07 | 12.65 |
| ***Anisodoris nobilis*** | | 3 | 88.91 | 1.66 | 5.24 | 0.74 | 5.85 |
| ***Cadlina luteomarginata*** | | 2 | 84.20 | 2.88 | 6.21 | 0.78 | 9.59 |
| ***Dendrodoris albopunctata*** | a,b | 9,9 | 78.30 | | 8.68 | | 13.02 |
| ***Hopkinsia rosacea*** | a,b | 7,7 | 76.70 | | 10.02 | | 13.28 |

**Appendix 2-B.** Organic content by body region of gastropod tissues.

Mean percentage organic content by wet weight (± standard error) of Foot, Mantle margin (Margin), Central mantle (Center), and Viscera.

| Species | N | Foot | Margin | Center | Viscera |
|---|---|---|---|---|---|
| Prosobranchs | | | | | |
| *Lirabuccinum dira* | 2 | 25.3 (0.3) | 24.1 (3.2) | 23.8 (0.2) | 24.8 (1.4) |
| *Nucella lamellosa* | 3 | 26.2 (1.2) | 26.3 (1.5) | 23.3 (0.7) | 30.1 (5.5) |
| *Tegula funebralis* | 2 | 25.7 (1.2) | 27.1 (2.8) | 24.6 (3.4) | 28.5 (4.7) |
| Unspiculated nudibranchs | | | | | |
| *Dendronotus iris* | 2 | 5.9 (0.1) | 4.4 (0.1) | 5.9 (0.7) | 14.8 (0.5) |
| *Dirona albolineata* | 3 | 4.6 (1.1) | 1.3 (0.1) | 4.4 (0.9) | 15.9 (1.5) |
| *Hermissenda crassicornis* | 3 | 12.4 (0.4) | 13.4 (1.3) | 12.5 (0.2) | 18.1 (1.0) |
| Spiculated nudibranchs | | | | | |
| *Acanthodoris hudsoni* | 3 | 6.2 (0.8) | 4.1 (0.4) | 5.2 (0.5) | 16.1 (1.8) |
| *Acanthodoris nanaimoensis* | 2 | 9.1 (0.5) | 5.7 (0.4) | 5.0 (0.4) | 12.6 (0.9) |
| *Aldisa cooperi* | 2 | 13.6 (1.1) | 9.8 (0.3) | 13.2 (0.0) | 23.4 (3.9) |
| *Archidoris montereyensis* | 3 | 12.0 (0.6) | 7.9 (0.5) | 7.4 (1.2) | 20.6 (1.4) |

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# CHAPTER 3

# NUDIBRANCH SPICULES: ANTIPREDATOR DEFENSE OR ENDOSKELETAL ELEMENTS?

## Introduction

Dorid nudibranchs and several other opisthobranch taxa such as notaspideans possess calcareous spicules (Hyman 1967), often present in fairly high quantities (Cattaneo-Vietti et al. 1993). This large investment suggests an important biological role, but spicule function has not been explicitly tested.

Spicules have often been considered a defense, perhaps because of nudibranchs' bright colors and shell-less form. However, no study has isolated the effect of spicules from antipredator chemicals (reviewed in Todd 1981, Foale and Willan 1987). Spicules could deter predators by several routes: by inhibiting digestive acids or enzymes, by increasing the effectiveness of chemical defenses, by increasing toughness, or by abrading gut linings (Hay et al. 1994). Spicules from other organisms are sometimes effective as deterrents alone (e.g., gorgonians) (Harvell et al. 1988, Van Alstyne et al. 1992) or in combination with chemical defense (Gerhart et al. 1988). However, deterrency sometimes varies among related species (Koh et al. 2000) and is perhaps ineffective against predators for some gorgonians and other taxa (Wylie and Paul 1989, Lindquist et al. 1992, Chanas and Pawlik 1996). With such variability, putative defensive roles require direct testing.

Alternatively, spicules could serve as structural support or as a true skeleton. Innumerable calcareous ossicles form an endoskeleton in sea stars (Eylers 1976) and

other echinoderms, and gorgonians also use spicules as supportive elements (Brusca and Brusca 1990). Nudibranch spicules certainly support external structures such as papillae (Garcia et al. 1986, Valdés and Gosliner 2001). However, we know little about their arrangement inside slug bodies, as most authors have looked only at surface conformations (Kress 1981, Foale and Willan 1987). Individual spicules are arranged in complex networks in at least one dorid nudibranch (Garcia et al. 1986). Characterizing these networks is crucial not only for their possible supportive role, but also as a potentially important phylogenetic character (Wägele and Willan 2000).

Defensive and structural roles are not mutually exclusive, and I tested both hypotheses using various approaches. Because a structural role would presumably lead to spicules being distributed approximately evenly within the body or in densities correlated with the complexity of overlying structures while a defensive role would lead to investment predominantly in the mantle, I investigated how spicules were invested throughout the body. I then directly tested deterrence to generalist predators via laboratory assays, and used whole mount and thin section staining to investigate internal arrangements of spicules, and their relations with other tissues.

## Methods

### Body content

Fifteen Cadlina luteomarginata (Nudibranchia: Doridacea) of 1.1 - 5.3 g wet weight were collected by hand, intertidally or subtidally using snorkeling or SCUBA from Barkley Sound, British Columbia. Animals were anesthetized in a 1:1 mix of 7% $MgCl_2$ to seawater (Smith and Carlton 1975), dabbed dry with a paper towel, and

weighed. They were then dissected into four external parts (rhinophores, gills, mantle, and foot) with the viscera removed. Each part was placed in an individual, pre-ashed, pre-weighed aluminum tray, and dried at 56°C for approximately 24 h to stable weight, measured via a 10-microgram balance.

Each sample was then incinerated in a muffle furnace at 500°C for 24 h to burn off organic tissue, and the remaining inorganic material weighed twice and averaged. This ash weight was taken as a reasonable proxy of spicule weight, because: a) inorganic salts comprise a fairly constant 3.5% of wet mass for most slugs (Chapter 2), b) organic, water, and ash contents do not significantly vary among parts for a given individual (B. Penney, unpublished data), and c) experimental removal of salts did not significantly affect results (B. Penney, unpublished data). Material was incinerated for 24 hours because visual inspection indicated residual organic material (charred black flakes adhering to spicules) after lesser intervals. There may be high loss of calcium carbonate at higher temperatures after this large amount of time (Paine 1971). However, the loss of $CaCO_3$ at approximately 500°C over 8 hours is minimal (A.R. Palmer, unpublished data). Data were compared three ways: a) as a regression of log total spicule weight versus log total slug dry weight, b) as a regression of log spicule weight versus log dry weight for individual body regions, and c) as a one factor ANOVA comparing the average ratio of spicule content to dry weight among body regions.

**Bioassays**

Predators

To test whether spicules are potentially deterrent to consumers, I chose for bioassays sympatric generalist predators known to eat molluscs. Cancer productus (red rock crab) and C. gracilis (graceful crab) are generalist predators on molluscs and other organisms (Orensanz and Gallucci 1988). Both overlap dorid nudibranch habitat in geography and depth (low intertidal to ca. 100 m from Alaska to California) and are large enough to attack adult dorids (Gotshall 1994). C. gracilis does not strictly overlap in habitat with C. luteomarginata, which is mostly found on hard bottoms (B. Penney, unpublished observations) and therefore served as a naïve predator to test for the generality of Cancer crab response. Individuals were collected either by baited trap or by hand using SCUBA from Bamfield and Grappler Inlets, Bamfield, B.C. Anthopleura elegantissima (aggregating anemone) is a sit-and-wait predator consuming large molluscs, such as Mytilus, that have been swept off rocks (Paine 1974). It is found from the intertidal to 20 m depth on rocks and other structures from Alaska to California (Gotshall 1994), and was extremely common at nudibranch collection sites. Individuals were removed from rocks by hand at Scott's Bay, Bamfield, B.C., and allowed to acclimatize to laboratory.

All predators were maintained in individual containers with flowing natural seawater at Bamfield Marine Station and fed every other day, initially with cracked-open Mytilus spp. (blue mussels), then with ca. 2 $cm^2$ pieces of squid mantle for 1 week before the experiment.

Bioassay design

Seventeen C. luteomarginata were collected using SCUBA from Scott's Bay, Bamfield, B.C. and anesthetized in a 1:1 mix of $MgCl_2$ and seawater. Viscera were then removed so only compounds contained in the body wall, and not those only present in undigested prey in the gut, would be in extracts. The remaining 117 g wet weight of flesh was thrice extracted with three times volume MeOH:$CHCl_3$ (2:1) for 24 h each time. This extract was reduced to a small volume of methanol under vacuum using a rotary evaporator.

To test effects of spicules alone or in combination with chemical defense, I added both spicules and extracts at natural concentrations to an artificial food recipe modeled after Lindquist and Hay (1996). The base artificial food was made from squid flesh and water (1:1) mixed with sodium alginate (2% of final wet weight) in a Waring blender. To this base, I added: a) either spicules at 4.6% of wet weight or not, or b) either methanolic extracts at 1 g nudibranch equivalent per 1 g of food or methanol alone. This formed four treatments: control (no additives), spicules alone, extracts alone, or spicules plus extracts. After ingredients were mixed thoroughly, the food was then solidified in a solution of 0.25 M $CaCl_2$ until it was approximately the consistency of cooked pasta. Because nutritional content can affect the acceptability of food to predators (Chapter 2), I ensured this recipe produced food at the natural nutritional quality of dorid nudibranch tissue (data not shown), approximately 12% organic content per unit wet mass.

Individual predators received each treatment once, at the rate of one treatment per day. The order in which treatments were presented to each individual determined

determined via a random number table; the number of individuals receiving each treatment on a given day was roughly similar. Prior to bioassays, each individual predator was tested with a small piece of squid flesh (ca. 1 $cm^2$) to ensure it was hungry before receiving experimental food. Food pellets were delivered directly to predator "mouthparts" (i.e. tentacles or chelae) and, if necessary, left in tanks for 2 h (crabs) or overnight (anemones). I recorded acceptance if > 75% of the food was consumed, or rejection otherwise. For anemones, I also recorded rejection for any piece spit out whole within the next 24 h, making this a conservative estimate of food acceptability. Each individual predator was only used once in each bioassay to avoid pseudoreplication, and the results of bioassays were analyzed via Cochran's Q test (Zar 1984).

Spicule Organization

One buffered formalin-preserved specimen of C. luteomarginata was chosen for clearing and staining using a modified ichthyology protocol as follows. The specimen was rinsed briefly in fresh water and transferred to 95% ethanol to remove remaining preservative, then dissected into foot, mantle, rhinophores and gills to allow better visualization. Viscera were not further treated, but were kept as vouchers. Samples were cleared in phenol over 2 d until translucent, then further digested in a stock alcohol-hydroxide solution (70% EtOH and 1% NaOH, mixed 15:1). Cleared samples were then stained with Alizarin red mixed at 0.03% by weight in the above alcohol hydroxide solution. Slight overstaining was removed

through 24 h treatment in the stock alcohol-hydroxide solution. Samples were then mounted in glycerol to further clear tissues.

Tissues were photographed through a Wild Stereomicroscope fitted with a Nikon DMX 1200 digital camera, and images captured using Nikon "Act1" software. Images of the mantle edge and rhinophores (Figs. 3-3A, 3-5B) are montages of several images taken with different focus, which were combined on Photoshop to create a picture with a better depth of field.

Histology

One C. luteomarginata was collected by hand using SCUBA in Grappler Inlet, Bamfield, B.C. in February 2001, anesthetized as above, then preserved in 10% buffered formalin over several days. After a brief rinse in tap water to leach out residual formalin, this specimen was decalcified in RDO for 5 h, rinsed again in tap water, and transferred to 70% EtOH. Tissue from the mantle edge was kept in 70% EtOH in a vacuum oven for several days, then further processed through 95% EtOH, 100% EtOH, 100% EtOH, Toluene, Toluene, and Paraffin using a Fisher Histomatic Tissue Processor, model 166, and embedded in labeled wax blocks using a Tissue Tek II machine. I used an American Optical 820 Spencer Microtome to cut 5 $\mu$m cross-sections, which were transferred to cleaned, labeled glass slides coated with 3% albumin/glycerol solution and dried on.

To visualize connective and muscle tissue, slides were stained with a Masson's trichrome recipe modified for marine molluscs (R. Mandryk, unpublished methods), then cleared in toluene and mounted in DPX under a glass coverslip.

Tissues were photographed through a Leica DM IRBE Microscope fitted with a Nikon DMX 1200 digital camera and processed using Nikon DMX "Act 1" software. However, images of cross sections were not combined or manipulated.

## Results

### Body content

Spicule weight (log ash weight) increased directly proportional to slug size (log total dry weight) for the whole animals (Fig. 3-1A) and for the mantle and foot (Table 3-1). Therefore, investment in the largest body regions did not change over the range of sizes investigated. However, both rhinophores and gills showed small but statistically significant departures from isometry (Table 3-1). Investment in spicules was heavy in some areas, e.g. up to 45% of dry weight in the mantle (Fig. 3-1B). Distribution of values for ash weight/dry weight was not statistically different from normal ($P = 0.633$, Shapiro-Wilk W test), and variances were homogenous (Levene test, F Ratio = 1.90, $p = 0.139$). Group means were significantly different (ANOVA, $p < 0.0001$), with significant differences between all pairwise comparisons (Fisher's PLSD, $p < 0.0001$) except Foot vs. Rhinophore ($p = 0.845$). Because the y-intercepts for each regression on untransformed ash weights were close to zero, a factorial ANOVA is acceptable to compare these ratios (Packard and Boardman 1988).

### Bioassays

Crabs were significantly deterred by nudibranch extracts (Cochran's Q, $p < 0.05$) but not spicules. The latter had little deterrent effect alone, and did not seem to

increase the effectiveness of chemical extracts (Fig. 3-2A, B). Both C. productus and C. gracilis responded similarly, suggesting that the outcome of bioassays was due to ingrained behavioral responses, not prior experience in natural habitats. Anemones were not deterred by spicules or extracts alone, but the combination of spicules and chemical extracts significantly reduced feeding with respect to control food (Cochran's Q, $p < 0.05$; Fig. 3-2C).

Spicule Organization

Spicules were regionalized to the cores of tracts made of connective tissue, where spicules were mostly parallel to the long axis of the tracts (Fig. 3-3A). These fibers formed an underlying reticulated network, from which issued thick vertical tracts, leading to loosely organized plushes of spicules supporting the papillae (Fig. 3-3C). Spicule tracts were more finely reticulated towards the edge of the mantle. Horizontal spicule tracts were not all on exactly the same plane, and this network was generally less dense through the central notum over the viscera (Fig. 3-3B). Magnifications up to 500X only revealed fusiform spicules (sensu Garcia et al. 1986); stellate spicules were absent.

Spicule networks in the foot were organized as in the mantle, but lacked the vertical tracts and papillae. The foot also had a more definite border of spicules (Fig. 3-4A) that was finer and more highly reticulated near its edge. Tracts were less dense near the midline of the foot, but continued uninterrupted all the way across its breadth (Fig. 3-4B). Unlike the mantle, tracts seemed to be mostly in the same horizontal plane.

Gill branches were supported by a fine axial skeleton, clearly organized into two rows of spicules up each main branch (Fig. 3-5A). Most spicules seemed oriented perpendicular to the axis of branch. The mantle around the gills was not significantly more densely spiculated than the rest of the mantle, but this ring was still supported by numerous vertical tracts leading to the surrounding papillae. The lamellae of the rhinophores were supported by dense rows of parallel spicules (Fig. 3-5B). The surrounding sheath was densely lined with spicules, but not clearly in ramified networks such as the rest of the mantle. Spicules did not extend through the shaft below the level of lamellae.

Histology

Thin sections confirmed several aspects of the Alizarin red staining. First, spicules are embedded in a connective tissue matrix (blue staining) in discrete frameworks. Horizontal tracts parallel the shape of the mantle, and vertical tracts extend into disorganized brushes that support papillae (Fig. 3-6A). Thin bands of muscle surrounding connective tissue follow the fibers in a manner analogous to circular or mantle retractor muscles, with some groups of bands possibly analogous to dorsoventral muscles. Close connections between muscle and spicules at various points indicate possible insertion points from the thicker plates of connective tissue (Fig. 3-6, B&C). The bottom edge of the mantle has a different structure than overlying areas, due to the short interspersed bands of muscle and connective tissue.

## Discussion

### Body Content

Over the range of body sizes tested, investment in spicules increased isometrically overall and for both the mantle and foot (Fig. 3-1A; Table 3-1), so the spicule investment in each part as a proportion of the whole was fairly constant. Rhinophores showed a slightly negative allometry, while gills showed a slight positive allometry in spicule investment (Table 3-1). These organs are significantly smaller than the mantle, and are more difficult to measure accurately, which likely accounts for the slightly lower $R^2$ values. However, the highly significant values for $P_{slope}$ in each case suggest a real departure from isometry. Both organs are more structurally complex than the mantle or foot (Fig. 3-3 through Fig. 3-5), and the relative requirements for support material versus flesh perhaps change with size. Another cryptobranch, Discodoris atromaculata, has a slightly negatively allometric investment in spicules (Cattaneo-Vietti et al. 1993).

Regardless, this pattern contrasts with the very strong positive allometry of shell mass seen in several prosobranch gastropods (Palmer 1981, and references therein) and some bivalves (Dame 1972, Hickman 1979), where adults produce less porous or more massive shells than juveniles. Skeletons of calcium carbonate can impose significant limitations on growth rate in many marine gastropods because soft tissue growth cannot exceed that allowed by available shell volume (Palmer 1981). However, in nudibranchs the spicules do not create a structure as restrictive as a shell, because spicules form separately within cells (Hyman 1967), allowing greater soft tissue per mass of calcium carbonate and increasing the surface area for mineral

deposition. This may partially alleviate limitations to growth rate imposed by such exoskeletal structures, which may be important given nudibranchs' often high growth rate. Perhaps there is no advantage to a proportionally greater investment in spicules at a larger size, or the cost of production is low enough (Palmer 1992) that smaller dorids can produce the same high proportion of spicules to body weight without suffering decreased growth rates. Most nudibranchs are presumed to be annuals or subannuals (Thompson 1967) but Cadlina laevis is perrennial and iteroparous (Todd et al. 2001). If costs of growth are important, these differences in life history may change the pattern of investment relative to other dorids. Due to difficulties in collection and weighing, it is unclear what happens in juveniles or smaller slugs. However, spicules are present at metamorphosis in Rostanga pulchra (Chia and Koss 1978) and shortly after hatching in Cadlina laevis (Thompson 1967).

The distribution of spicules in tissues was consistent with both defensive and supportive roles. The mantle contained the greatest proportion of spicules, up to 45% of dry weight (Fig. 3-1B). As the mantle is the region first encountered by predators, this supports the hypothesis of spicules being arrayed for defensive purposes. Although not quantitatively measured, staining revealed much denser spiculation in the mantle edge – where predators would first bite (B. Penney, pers. obs)– than in the central mantle (Fig. 3-3A). However, concentrations in other tissues were quite high (up to 35% of dry weight), even in regions such as the rhinophores that would be withdrawn during predator attack. A similar pattern has been found in Discodoris atromaculata (Cattaneo-Vietti et al. 1993). This could be because the rhinophores' chemosensation role makes them more important to protect, or because of the

structural role of spicules in the rhinophores. The gills have the lowest percentage of spicules per dry weight, perhaps because more space is needed for circulatory and respiratory functions. Regardless, the higher investment of spicules in mantle edge tissue of C. luteomarginata supports the hypothesis of antipredator defense, while high concentrations body-wide, even in areas withdrawn when the slug is disturbed, supports a structural role.

Bioassays

Nudibranch spicules are unlikely an important defense against generalist predators. In bioassays, spicules did not significantly deter crabs, either alone or when added to chemical defense (Fig. 3-2A,B). Interestingly, C. gracilis responded the same as C. productus in assays. Because C. gracilis wouldn't often encounter C. luteomarginata in its natural habitat, this suggests that C. luteomarginata extract is defensive against some crab taxa, regardless of previous experience.

Anemones were not deterred by spicules alone, but the combination of spicules and chemical extracts significantly reduced feeding relative to control food (Fig. 3-2C). Unfortunately, this experimental design precludes specific testing for the synergistic effects suggested by these results. The lack of extract deterrence compared to that of whole slugs (Appendix II) may indicate that some deterrent compounds were lacking in the final mixture. Anemones posed an extra complication because they sometimes spat out food pieces several days after tests. However, because a whole nudibranch would presumably be dead after such time, I still counted these pieces as "consumed."

Do these results represent acceptability to other potential predators? Potential nudibranch consumers include specialized opisthobranchs, fish birds, and perhaps sea stars (Todd 1981). Navanax, a specialist predator on opisthobranchs, seems to avoid nudibranchs with spicules (Paine 1963). However, extra stiffness due to spicules is probably not the cause, as Navanax willingly consumes shelled bullomorphs (Harris 1973). Tidepool sculpins (Oligocottus maculosus) respond similarly to anemones with respect to spicules and chemical extracts (G. Lichota, unpublished results). To my knowledge, potential bird predators have not been tested. However, given that gulls eat sea stars and regurgitate the calcareous elements, and crows can peck through outer tissues to access nudibranch viscera (Appendix II), birds likely aren't deterred by spicules alone. Likewise, tests with sea stars (Pycnopodia helianthoides) were inconclusive, but suggested no deterrence by spicules (B. Penney, unpublished data). Even if spicules do not behaviorally deter predators, they may increase toughness (Koehl 1982). However, many fish, anemones, and predatory opisthobranchs swallow prey whole, crabs tear off pieces, and birds swallow prey whole or peck them apart. Increased toughness, at least within the range achievable for sea slugs, probably does not matter for defense against these predators. Support for spicules as a defense is therefore fairly weak, although we cannot rule out synergistic effects with chemical defense against some predators.

The defensive effectiveness of spicules for other organisms seems to vary considerably. Gorgonian soft corals are the most extensively varied in this regard, and spicules from Pseudopterogorgia, Gorgonia, and Sinularia deter predators (Harvell et al. 1988, Van Alstyne et al. 1992). However, for Leptogorgia virgulata,

only a combination of extract and spicules significantly deterred pinfish (Lagodon rhomboides) (Gerhart et al. 1988). Not all alcyonarian sclerites are deterrent at natural concentrations (see Van Alstyne et al. 1992, Puglisi et al. 2000 for discussion), or to all predators, as sclerites are of little importance in determining the feeding preferences of the butterfly fish Chaetodon unimaculatus (Wylie and Paul 1989). Deterrency of spicules also seems to vary among gorgonian speices (Koh et al. 2000). Small stellate spicules from a tropical didemnid ascidian did not deter fish feeding (Lindquist et al. 1992). Sponge spicules, long thought a major form of defense, are not effective against reef fish (Chanas and Pawlik 1995) or sea stars (Waddell and Pawlik 2000), and are likely ineffective against sponge specialists (Chanas and Pawlik 1995, and references therein). Therefore, spicules may sometimes serve as a defense, but it is unclear which kinds, against which predators or under what circumstances. Possible contributing factors include the type of defense offered (mechanical, mineral, toughness), predator adaptation (e.g., degree of mechanical shielding), and synergisms with other defenses (reviewed in Hay et al. 1994). Regardless, this lack of effectiveness against predators seems surprising, as cryptobranch dorids lacking spicules often have more potent chemical defenses, suggesting some sort of trade-off in investment (Cimino and Ghiselin, 1999).

Organization

Spicules in C. luteomarginata were organized into ramified networks, embedded in connective tissue, which support external structures such as rhinophores, gills and papillae. This network is dimly visible through the underside of the mantle

(MacFarland 1966). The one other detailed survey of spicule networks – on Doriopsilla areolata (Nudibranchia: Doridacea) (Garcia et al. 1986) – reveals a pattern similar to C. luteomarginata. Although these species are not closely related (Valdés and Gosliner 1999), they have similar features. Mantle spicules lie parallel to the surface and are organized into a network of tracts with almost rhomboidal lumens in between (Fig. 3-3A). Vertical tracts arise from this network (Fig. 3C) to form disorganized plushes inside papillae. Vertical tracts, and the form of spiculation inside papillae, are similar in both species. Spiculation in both species is thinner in region inside where body wall meets the mantle (Fig. 3-3B). In peripheral regions, horizontal tracts form more than one plane in places, although in C. luteomarginata more than one plane can be seen even near the edge of the mantle.

Spicule networks in other body regions seem to show more variation between these species. In both, the foot has networks similar to the mantle, but lacking vertical tracts, thinner and more ramified near edges, and with a fairly distinct border as compared to mantle (Fig. 3-4A). However, in C. luteomarginata the tracts in the foot are not so clearly perpendicular to the longitudinal body axis as in D. areolata. Also, in D. areolata the tracts disappear near the midline of the foot, whereas the pedal network in C. luteomarginata is thin but continuous through the midline (Fig. 3-4B). C. luteomarginata gills are more heavily spiculated, have spicules further up the gill branches, and are more clearly organized into tracts up each branch compared to D. areolata (Fig. 3-5A). Rhinophore sheaths are heavily spiculated in a pattern similar the to the rest of the mantle in both species, and the rhinophores themselves have parallel rows of spicules holding out the lamellae (Fig. 3-5B). However, C.

luteomarginata lacks spicules in the basal third of the rhinophore shaft. Lastly, I did not find stellate spicules in C. luteomarginata even at 50 X magnification, although I did not specifically digest tissue for them. However, this spicule class may be present in a congener, C. flavomaculata (MacFarland 1966). These differences suggest at least some degree of variation in spicule networks among taxa, making them a promising phylogenetic character once their form has been characterized for more taxa (Wägele and Willan 2000).

Histology

Thin sections confirmed several findings from whole-body stains. Spicules are embedded in tracts of connective tissue, both horizontal tracts out to the mantle edge and vertical tracts extending into slight plushes supporting papillae (Fig. 3-6A). Connective tissue sheaths around spicule tracts may be a common feature, based on other investigations (Hyman 1967, Garcia et al. 1986). Only one horizontal spicule tract is visible in each section, suggesting that the multiple levels seen in whole mounts are from horizontal tracts extending at different heights. The plush of spicules supporting papillae are not obviously wider than the vertical tracts of spicules supporting them, unlike in whole mounts where papillae appear wider. This may be from variation among individuals, or some slight tissue distortion due to preservation or processing.

Could spicule networks serve as muscle antagonists? Key evidence is whether muscles attach to the spicules or connective tissue of these networks, as opposed to just the body wall or other muscles, as seen in other molluscs (Fretter and

Graham 1962, Hyman 1967) and non-spiculated opisthobranchs (Lemche 1956). Muscle bands closely followed the connective tissue/spicule network (Fig. 3-6A), and had close connections to it, similar to insertion points (Fig. 3-6B,C). Similar muscle attachments are found into spicules in caryophyllidia of Rostanga arbutus, Jorunna sp., and other species (Kress 1981, Foale and Willan 1987) and possibly retract these structures, although this has not yet been observed in living specimens. Muscle attachments were not discussed by Garcia et al. (1986) for D. areolata, so the universality of this arrangement is unclear.

However, the overall pattern of musculature is similar to the mantle and infrapallial retractors seen in other gastropods. Several prosobranchs have mantle retractor muscles that branch analogously to the horizontal fibers seen in C. luteomarginata, although the smaller branches are not shown in these diagrams (Fretter and Graham 1962). Cylichna is one of the few opisthobranch taxa with extensively studied mantle musculature (Lemche 1956); however, its shelled form and thin mantle preclude direct comparison. No longitudinal muscles were seen in mantle edge cross sections, but this is likely because these muscles lie elsewhere. Diaulula sandiegensis contains dorsolateral longitudinal muscles in the mantle on either side of the visceral cavity– probably analogous to pedal retractors– which extend into the foot where they are highly integrated with the tarsos musculature (Uyeno 1999). Uyeno felt that this tarsic musculature inserts into the walls of the foot and mantle skirt wall; thus, this musculature may be functionally associated with the spicular network (Uyeno, pers. comm.). The actual points of attachment, and whether these vary among dorid taxa, remain to be investigated. There are currently few other

data on musculature in spiculated opisthobranchs for a more general comparison (Hyman 1967, Gosliner 1994).

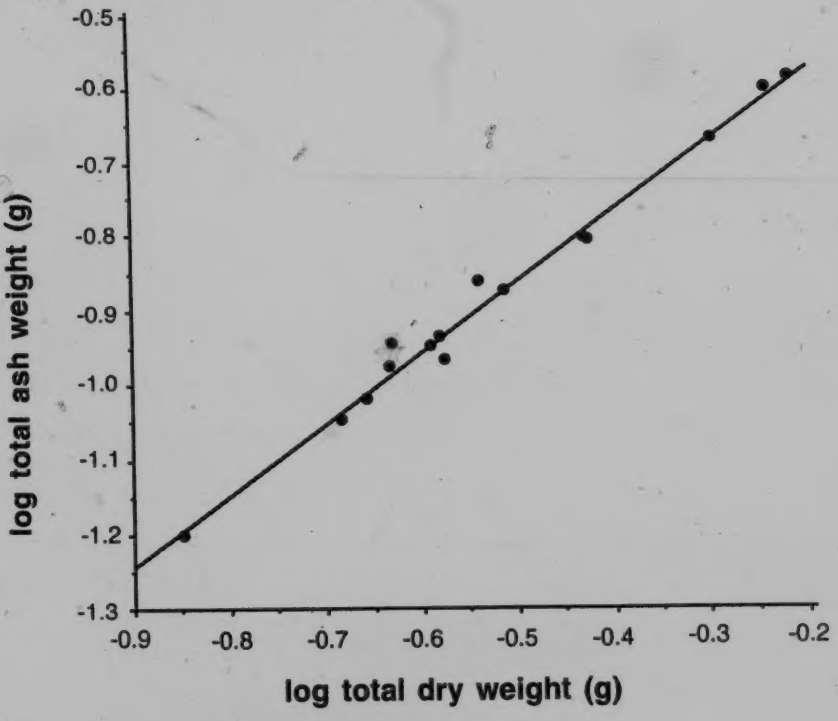
Other invertebrates such as echinoderms possess multi-part endoskeletons of calcium carbonate with attached musculature, but such constructions have not previously been noted for sea slugs (Brusca and Brusca 1990). If this network constitutes a skeleton, it is certainly of unusual form, as the spicules don't directly articulate against each other and there are not specific sets of muscles linked to individual hard units. An alternate model is that the spicules stiffen the connective tissue tracts to form an unbraced framework that provides extra stiffness for muscle antagonism (Vogel 1988). Given the relative cost of calcium carbonate versus protein (Palmer 1983), a mixed connective tissue and spicule antagonist may be cheaper to produce than connective tissue alone. The large hemocoel of slugs would generally allow faster movement than a muscular hydrostat, but but potentially sacrificing regional control (Uyeno 1999, and references therein ). Having a solid framework with discrete muscle attachments may allow for more regionalized control of motion. Given the available evidence, this is perhaps a more plausible raison d'être for spicules than antipredator defense.

**Table 3-1**. Spicule investment in Cadlina luteomarginata. N = 15 for all comparisons. Least-squares regression equations for whole body dry weight (or log(whole body dry weight)) versus various body regions. S.E., Standard Error; Adj. $R^2$, adjusted $R^2$; P = exact probability; RMA, Reduced Major Axis. $P_{slope}$ indicates the probability that the regression slope equals zero. $P_{allom}$ indicates the probability that the slopes conform to an isometric relationship (i.e. one-sample t-test against 1.0).

| Body Region | Units | Slope (± S.E.) | Intercept | Adj. $R^2$ | $P_{slope}$ | $Slope_{RMA}$ | $P_{allom}$ |
|---|---|---|---|---|---|---|---|
| Whole body | Ash | 0.418 ± 0.011 | 0.006 | 0.991 | 0.0001 | | |
| | Log ash | -0.959 ± 0.030 | -0.038 | 0.986 | 0.0001 | 0.965 | 0.2600 |
| Mantle | Ash | 0.353 ± 0.011 | 0.006 | 0.987 | 0.0001 | | |
| | Log ash | 0.949 ± 0.035 | -0.452 | 0.982 | 0.0001 | 0.958 | 0.2500 |
| Foot | Ash | 0.060 ± 0.003 | -0.001 | 0.963 | 0.0001 | | |
| | Log ash | 1.017 ± 0.052 | -1.231 | 0.965 | 0.0001 | 1.035 | 0.5119 |
| Gill | Ash | 0.003 ± 0.000 | 0.000 | 0.931 | 0.0001 | | |
| | Log ash | 1.320 ± 0.111 | -2.466 | 0.909 | 0.0001 | 1.379 | 0.0042 |
| Rhinophore | Ash | 0.001 ± 0.00 | 0.000 | 0.798 | 0.0001 | | |
| | Log ash | 0.713 ± 0.101 | -2.861 | 0.778 | 0.0001 | 0.800 | 0.0131 |

**Figure 3-1**. Spicule content of individual Cadlina luteomarginata. A. Regression of log spicule (ash) weight versus log dry weight (n = 15). B. Spicule content by body region. Bars sharing letters within each panel were not significantly different (n = 15; ANOVA, Fisher's PLSD, $p < 0.05$).

**A.** Spicule content by weight




**B.** Spicule content by body region

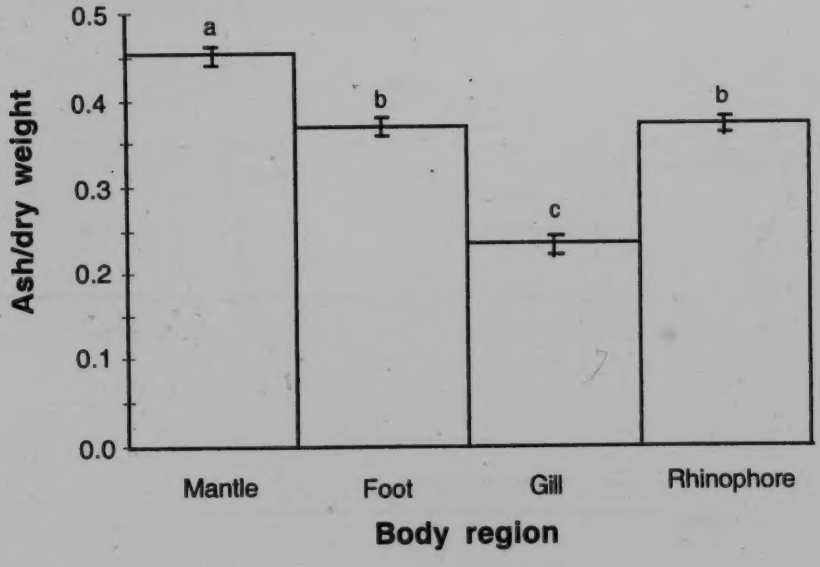


**Figure 3-2**. Bioassay results of spicules and chemical extracts with generalist predators. A. Cancer productus (n = 13), B. Cancer gracilis (n = 14), C. Anthopleura elegantissima (n=14). Treatments: C = control, S = Spicules, X = Extract, SX = Spicules plus extract. Bars sharing letters within each panel were not significantly different (Cochran's Q test, $p < 0.05$).

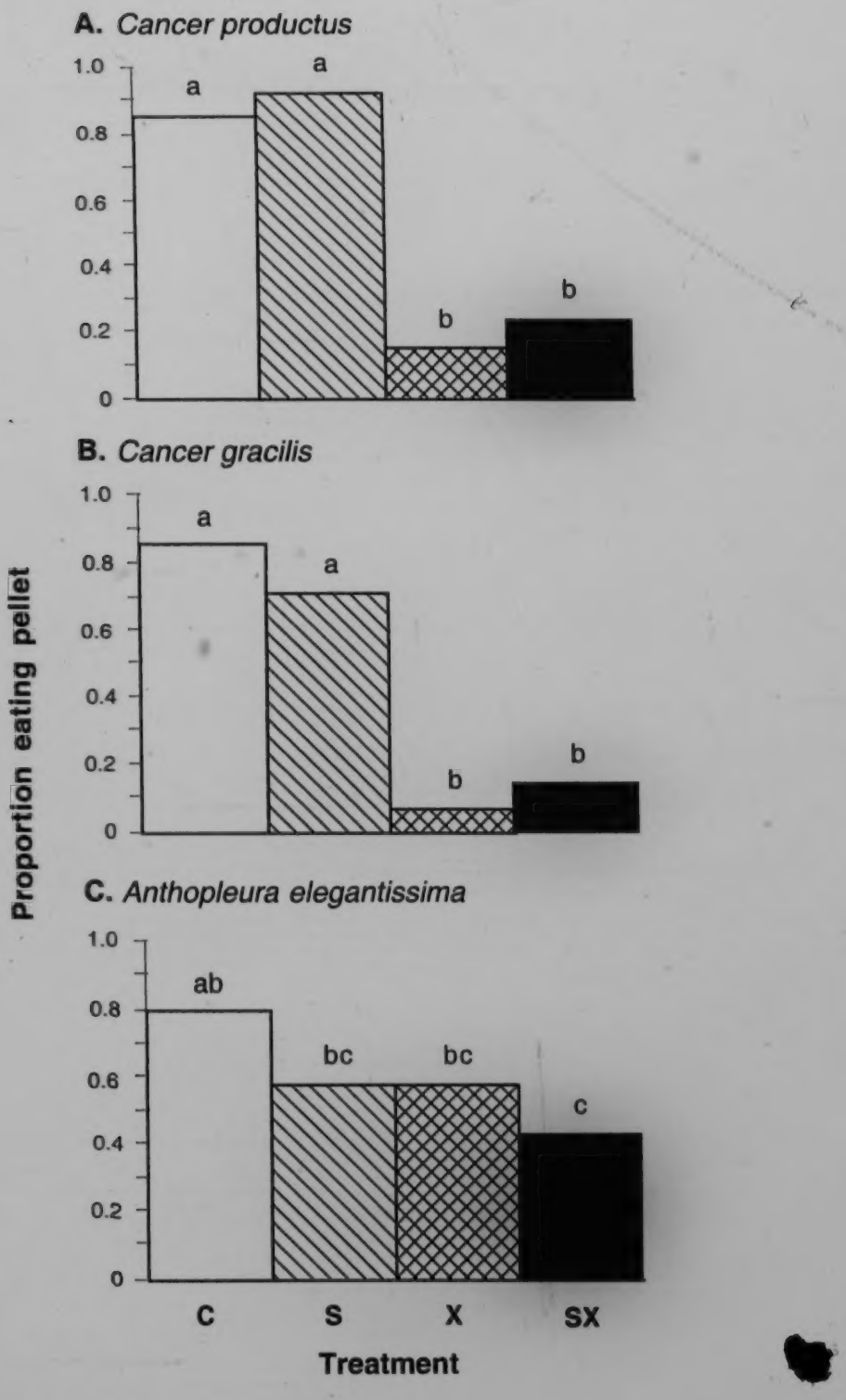

A. Cancer productus
1.0
0.8
0.6
0.4
0.2
0
a
a
b
b
B. Cancer gracilis
1.0
0.8
0.6
0.4
0.2
0
a
a
b
b
Proportion eating pellet
C. Anthopleura elegantissima
1.0
0.8
0.6
0.4
0.2
0
ab
bc
bc
c
C
S
X
SX
Treatment

**Figure 3-3**. Spicule staining: light microscopy of Cadlina luteomarginata mantle. A: Mantle edge, dorsal view. Anterior-posterior axis is approximately horizontal (bar = 500 μm). B: Central mantle, dorsal view, showing vertical tracts and spicule plushes supporting the papillae. Anterior-posterior axis is approximately vertical (bar = 500 μm). C: Vertical tracts supporting papillae surrounding gill ring, dorsal view. Anterior is to the top of the image (bar = 100 μm).

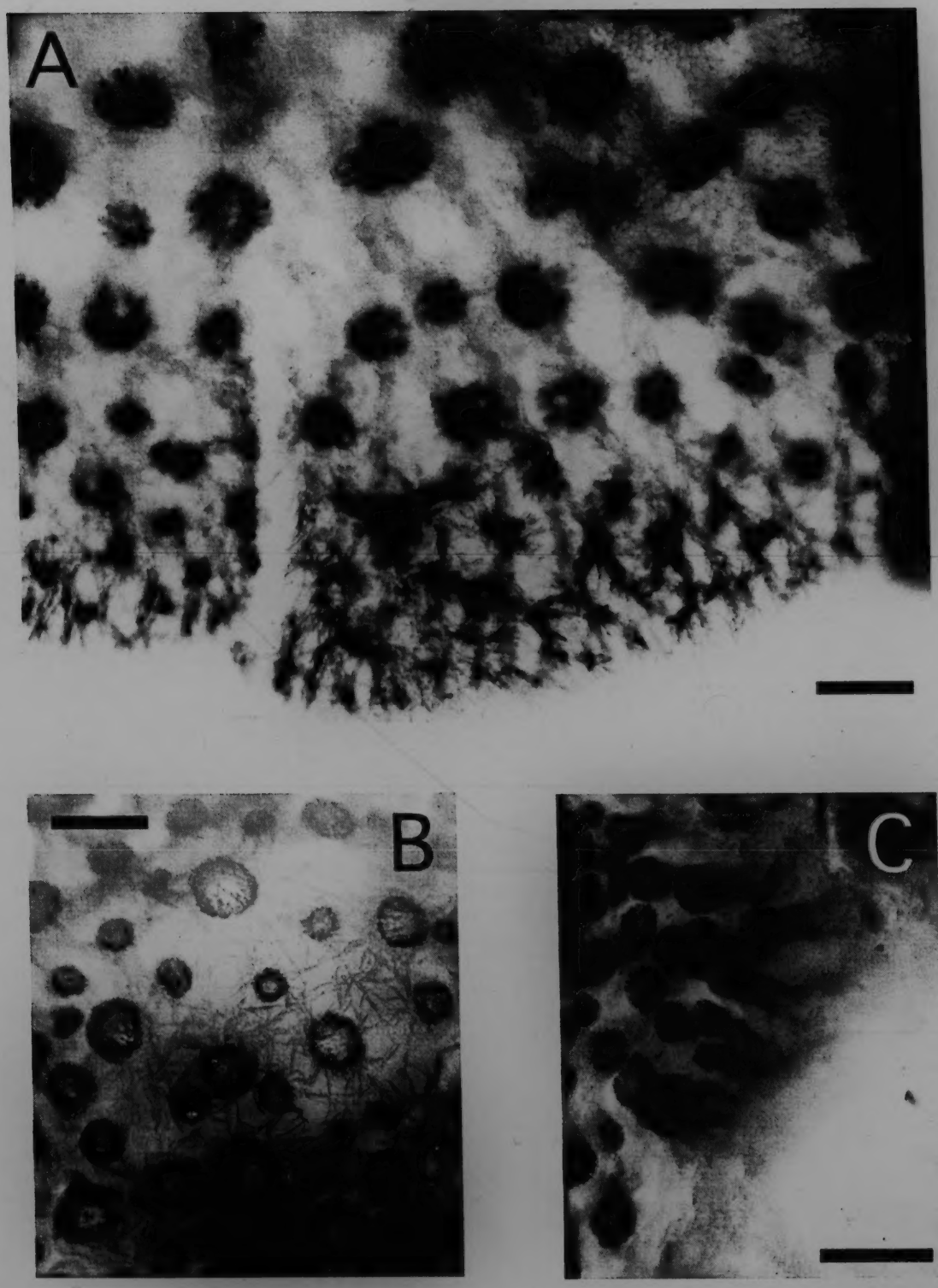

A
B
C

**Figure 3-4**. Spicule staining: light microscopy of Cadlina luteomarginata foot. A: Edge of foot, ventral view. Posterior of the specimen is to the top of the image (bar = 250 μm), B. Foot center, ventral view. Anterior-posterior axis is approximately vertical (bar = 100 $\mu$m).

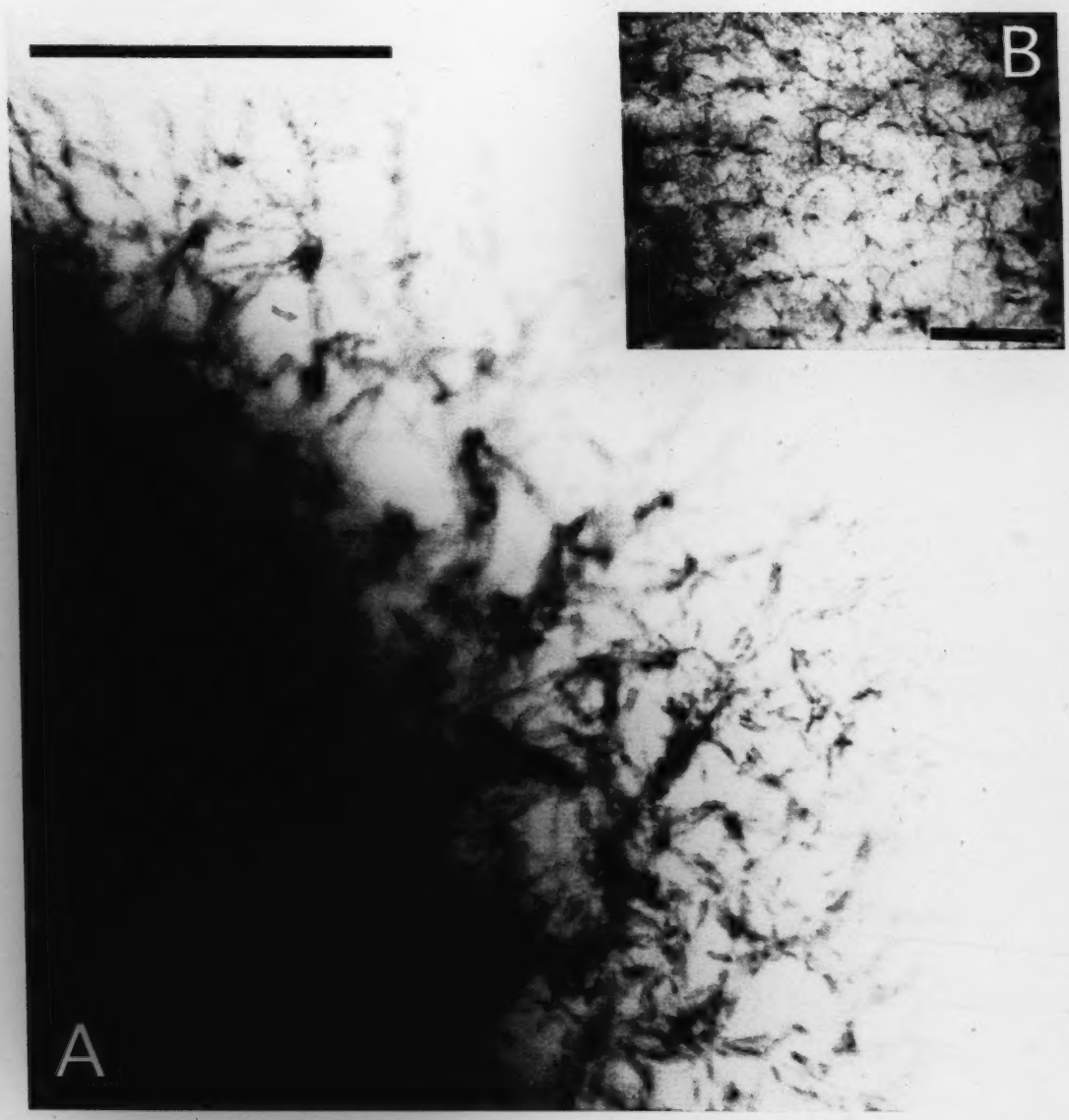

B
A

**Figure 3-5**. Spicule staining: light microscopy of Cadlina luteomarginata external structures. A: Gills (bar = 250 μm), B: Rhinophores (bar = 1000 μm).

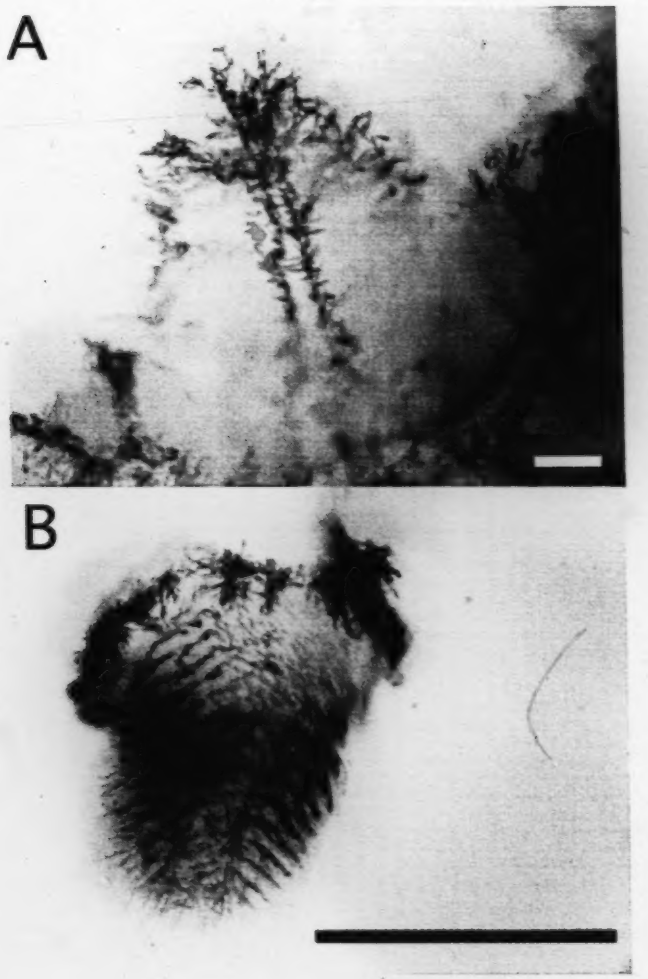
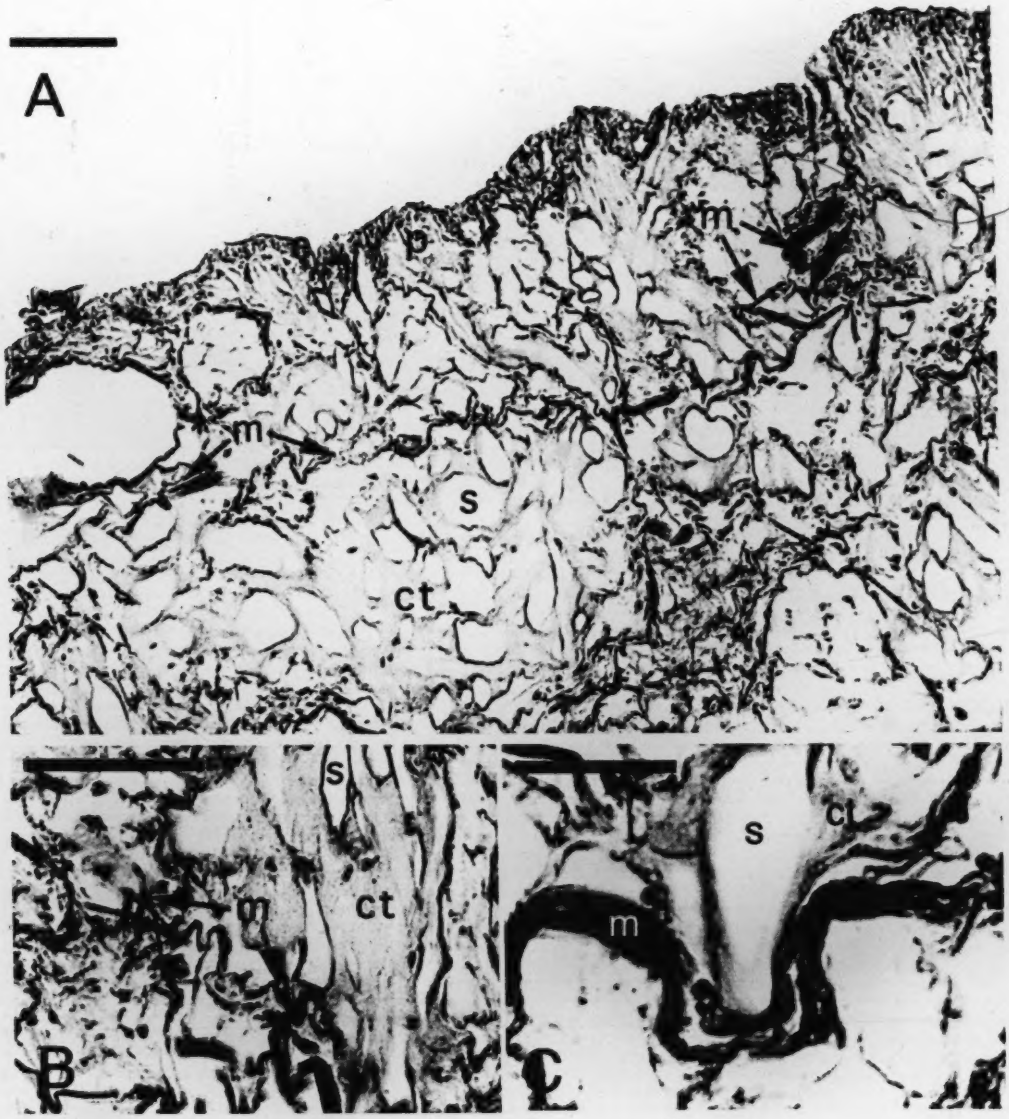

A
B

**Figure 3-6**. Cadlina luteomarginata: Light microscopy of connective tissue and musculature in mantle cross-sections. A: Top of mantle, approximately halfway between the body wall and mantle edge, showing papillae and underlying structure. B and C: Muscle insertion into spicule/connective tissue matrix. ct: connective tissue; e: epidermis; m: muscle bundle; p: papilla; s: spicule. Scale bars = 50 μm.


A
m
s
ct
m
s
ct
B
s
ct
m
C

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# CHAPTER 4.

# PREY USE BY TWO NORTHEASTERN PACIFIC CRYPTOBRANCHIA (NUDIBRANCHIA: DORIDACEA). ARE THEY AS "SPECIALIZED" AS WE THOUGHT?

## Introduction

Feeding specialization seems less common in marine communities than in terrestrial ones (Lubchenco and Gaines 1981, Hay and Steinberg 1992). Insect herbivory has large impacts on terrestrial plants, and most phytophagous insects consume plants from only one or a few families (Bernays and Graham 1988). In contrast, most marine herbivores are fairly generalized in their feeding (Hay and Steinberg 1992). The bulk of marine specialists appear to be mesograzers – sedentary consumers much smaller than their prey – that rely on prey defenses for protection against large generalist consumers that eat both plants and their mesograzers (Hay and Steinberg 1992, Duffy and Hay 2001). For instance, the amphipod Pseudamphithoides incurvaria builds domiciles out of Dictyota, a brown alga that is well defended chemically, and these domiciles are its primary line of defense against omnivorous fishes (Stachowicz 2001). However, we lack data on two critical points to extend this model. First, marine environments have not been sampled as extensively as terrestrial ones; given that specialists are often less numerous than generalists, we may have missed examples of marine specialists. Second, many marine communities are dominated by sessile animals rather than plants (Nybakken

1997). Diets of carnivorous grazers, and the factors affecting them, have been largely unexplored.

Nudibranchs are "among the best-known marine specialists" (Paul 1992), and are largely carnivorous grazers on sponges, bryozoans, cnidarians and ascidians (Todd 1981). Many show striking adaptations to particular prey, including mimicry (Thompson 1976) and sequestration of prey chemicals or nematocysts (Avila 1995). One of the larger and most spectacular groups are the cryptobranchiate dorids, a monophyletic group (Valdés and Gosliner 2001) that feed almost exclusively on sponges (Todd 1981). These slugs have provided novel systems for natural products research (Cimino et al. 1999) and have been proposed as a marine system with which to test theories of feeding specialization based on phytophagous insects (Karuso 1987). Indeed, use of prey-derived defensive chemicals is often cited as the driving force in the evolution of this taxon (Cimino and Ghiselin 1999).

However, many common cryptobranch species in the Northeastern Pacific may not be feeding specialists at all. Numbers of reported prey species are large: for example, over thirty species have been reported for Cadlina luteomarginata (McDonald and Nybakken 1997). These wide prey ranges may be an artifact of anecdotal reporting, or failing to distinguish between slugs using species as substrate versus food. Yet many of these nudibranchs do not fit a typical 'mesograzer' profile. They are mobile enough to move among patches of prey and may have other defenses such as biosynthesized defensive chemicals (Avila 1995), low nutritional quality (Chapter 3), and to some degree calcareous spicules (Chapter 3). The few existing quantitative studies of cryptobranch diets from this region suggest broad ranges of

acceptable sponge prey (Bloom 1981, Hellou et al. 1982, Thompson et al. 1982). Without information on sponge abundance in the field, we cannot be certain these nudibranchs are even selective feeders, much less specialized.

Determining slug diet breadth is crucial to understanding the dynamics of many subtidal communities, many of which are spatially dominated by sponges (Dayton et al. 1974, Knowlton and Highsmith 2000). Nudibranch consumption can often greatly impact sponge biomass (Dayton et al. 1974, Bloom 1981, but see Barnes and Bullough 1996, Knowlton and Highsmith 2000), and has apparently contributed to several sponge-bivalve protective mutualisms (Bloom 1975, Pond 1992). To test whether these Northeastern Pacific cryptobranchs are selective feeders, I surveyed three sites in Barkley Sound, British Columbia for both sponge abundance and slug prey use. I combined these data with other quantitative field studies to show that, although slug foraging is significantly different from sponge abundance, the pattern of prey use does not fit typical definitions of specialization (monophagy or stenophagy). Most likely these nudibranchs are somewhat generalist sponge feeders, that avoid heavily defended species.

## Methods

### Study Sites

To identifiy areas where slugs were abundant, I surveyed approximately ten sites in the western part of Trevor Channel (Barkley Sound, British Columbia, Canada) in summer 1999. These preliminary sites were chosen based on wave exposure, location, and anecdotal reports. Of the sites with good numbers of

nudibranchs, I chose three that represented the range of communities found (Figure 4-1). Dixon Reef (DXR: 48° 51' 24" N, 125° 07' 06" W) is a rock reef approximately 100 meters in diameter and within 400 meters of shore and smaller reefs with kelp and mussels, separated by a sedimentary bottom in between. Despite its location well within Barkley Sound, its lack of western protection gives it medium wave exposure (Arsenault et al. 2001). The reef itself is mostly bedrock and boulder with a few areas of cobble. Scott's Bay (SB: 48° 50' 06" N, 125° 08' 48" W) was the least wave-exposed site, due to its northerly aspect. Sampling sites were mostly inside the protected area of the bay. The substrate varied from bedrock to large boulders with some cobble areas, and grades from kelp beds in shallow depths (ca. 4 m) to open rock and overhangs to a sandy bottom with low slope at ca. 12 m depth. Seppings Island West (SW: 48° 50' 30" N, 125° 12' 30" W) was the most wave exposed, with sampling sites on the west side of the island, in a bay facing northwest. The predominant substrate was open bedrock, with occasional boulders and cobble. Kelp beds were further away horizontally than at SB. The rock bottom is continuous with several reefs and shallower areas.

All sites were observed to have similar densities of generalist molluscivores: sea stars (Pycnopodia helianthoides and occasional Solaster sp.), fish (greenling and rockfish), and large crabs (primarily Cancer sp.) and there were no known specialist opisthobranch predators at these sites. Therefore, although predators on dorids at such sites (Todd 1981) are not well known, differential predation would not affect slug prey use among these sites.

Field Sampling

Sampling to determine sponge availability and use by nudibranchs was accomplished in late summer and fall of 1999. I used belt transects at circa 10 m depth because this depth seemed to adequately represent the shallow subtidal community while allowing reasonable time for surveys. For sponge abundance, I sampled 0.25 $m^2$ square quadrats at random distances along the transects (fin beats were predetermined by a random number table (Sokal and Rohlf 1981)). For each sponge patch encountered in quadrats, I recorded its identity and directly measured its area with a ruler. The efficiency of this sampling design was not directly assessed; however, 0.25 $m^2$ quadrats are a useful size for benthic organisms distributed approximately similar to medium-sized macrophytes (Pringle 1984). For any sponge not readily identifiable in the field, a sample was transferred to a coded mesh-bottomed vial and returned to the lab for identification. For simplicity, if any sponge patch was within 1 cm of another patch of the same species, they were counted as the same patch. Because the oral tentacles of these nudibranchs generally spans this distance (BKP, pers obs), this possibly reflects what they would detect as one patch. I collected any cryptobranchiate dorids found within 1 m of each transect (4-5 per site). Nudibranchs were returned to Bamfield Marine Station for fecal collections, after which they were returned to their original field sites. Sites were sampled until > 30 quadrats were complete and, if possible, over 15 individuals each of at least 2 species of slug had been collected.

Specimen Identification

Sponge samples were stored in flowing natural seawater at Bamfield Marine Station in mesh-bottomed vials until identification. Hand-cut sections of samples spanning inner and outer regions of the sponge were studied under a dissecting stereomicroscope, digested in bleach, and their skeletal structures sketched. Further bleach digestion freed spicules, which were sketched and measured (n= 20-50 of each type) using a compound microscope with attached camera lucida and digitizing tablet. Data were recorded using MacMeasure II 2.33 (Rasband and Palmer 1993) and calibrated with a stage micrometer to generate a length frequency distribution for each spicule type. Samples were then identified using standard references for the Northeastern Pacific (Smith and Carlton 1975, Kozloff 1996). Identification of questionable species were confirmed by Drs. William Austin and Curt Smecher if possible, but otherwise were assigned to labeled OTUs (see Table 4-1).

To collect fecal samples, slugs were kept without food in 1 L mesh-sided tupperware cages in flowing seawater at Bamfield Marine Station for several days. Feces were collected using a clean Pasteur pipet, and transferred to labeled vials until identification. Randomly selected samples from 10-15 individual slugs of each species were chosen from each site, if available, as preliminary results showed these numbers were adequate to obtain measures of selectivity achievable by the whole set of collected slugs.

For identification, feces were smeared on a clean glass slide and observed under a compound microscope. Any spicules, fibers, or other material were sketched and noted. Samples were then digested with bleach to obtain spicules, which were

measured and sketched as described above. Slugs were classified as "empty gut" if samples had too few spicules to identify accurately; these samples often contained fewer than 10-20 spicules per slide (representing approximately 1-5% of collected fecal volume), and most of these were broken or represented mixes that did not occur in any known sponge.

Identifications of sponge prey were made by comparing the spicule profile in fecal samples to the sponges present at each site. In the few cases where fecal spicule profiles did not match any sponge present, spicules were identified as above for sponge samples. Sponge prey were considered identified if a unique spicule type was present (e.g., diancistras in Zygherpe hyaloderma) or if spicule morphology and size distribution matched for several spicule types of a particular sponge (e.g., acanthostyles and tylotes for Myxilla incrustans). Often, the spicule profiles were best explained by the presence of two or more sponge species, but a few samples did not match spicule profiles of sponges present at field sites or described in the literature, and were recorded as "unknown." Because some sponges have particular spicule types restricted to the basal mat (Smith and Carleton 1975), identification of prey via fecal samples could be problematic if slugs only grazed the surface of the sponge. However, observation of feeding in the lab and field suggested that slugs often grazed patches of sponge down to bare substrate (B. Penney, personal observation).

Two prey groups previously reported for C. luteomarginata (McDonald and Nybakken 1997) could not be accurately detected with this protocol because they lack spicules. Aplysilla spp. are potentially identifiable by the presence of distinct fibers in undigested samples (Linington and Penney, unpublished data), but were excluded

from consideration because this ensured greater comparability with other studies. Halisarca spp. lack both spicules and fibers, and are essentially undetectable in feces. Neither sponge was found at any of the field sites, but diet diversity for C. luteomarginata may represent an underestimate without these species.

These data on prey use therefore represent "isolations," or frequency of occurrence. This measure is useful when it is difficult to obtain data on diet composition by proportional volume (Hyslop 1980). Unfortunately, it is difficult to determine the relative volume of prey in each slug's diet from fecal spicules alone: relative spicule content to sponge volume, loss of spicules through digestive tract over time and variation with time of digestion are unknown and likely vary among prey species. This measure does not describe the diet of an individual animal, but shows how uniformly the population of predators selects a prey item without actually indicating importance with respect to other prey (Tirasin and Jørgensen 1999). As long as there are no individuals with an unusually high number of prey species in their feces, this is sufficient for matching observed diets against prey abundance. However, just as measures of percent cover can exceed 100%, so the number of "isolations" can exceed the number of individuals sampled.

Richness and Diversity Measures

Several measures of sponge community richness were performed to ensure sampling adequately reflected the available richness and diversity of demosponge prey (Begon et al. 1990), and to describe differences among sites quantitatively so as to explain potential differences in selectivity. Any specimen identifiable by field guides

was counted as a separate species, even if the species was not officially described. Likewise, some specimens were unclassifiable using current guides; similar specimens were lumped together, and each operational taxonomic unit (OTU) regarded as an "unknown species." These were counted as separate species for both richness and diversity measures to avoid underestimating these variables. For comparability to other studies, I calculated sponge diversity using the Shannon Index (Begon et al. 1990). Because it is unclear what comprises a sponge "individual" (Brusca and Brusca 1990) and because grazing slugs are partial predators (Harvell 1984), the proper measure of a single "prey item" is somewhat subjective. Therefore, I calculated diversity using both number of individual patches and area as measures of sponge abundance.

Prey Use and Selectivity

To compare abundance of sponges with use by nudibranchs, I used Pearre's index, a 2 x 2 $X^2$ based measure of selectivity. This index's advantages include simplicity of interpretation (-1 to 1 range, values above 0 mean positive selection, values below 0 mean avoidance), statistical ease, and applicability to small sample sizes (Pearre 1982). Selectivity (C) is calculated using the equation:

$$C = \pm \left[ \left( \left| a_d * b_e - b_d * a_e \right| - n/2 \right)^2 / (a_t * b_t * d_t * e_{tt}) \right]^{0.5}$$

Where, for the potential prey species of interest, A, and all other species combined, B:

| | Prey Species | | |
|---|---|---|---|
| | A | Others | Total |
| Diet | $a_d$ | $b_d$ | $a_d + b_d = d_t$ |
| Environment | $a_e$ | $b_e$ | $a_e + b_e = e_t$ |
| Total | $a_d + a_e = a_t$ | $b_d + b_e = b_t$ | $a_d + a_e + b_d + b_e = n$ |

These values readily lend themselves to testing against a $X^2$ distribution with one degree of freedom, with $X^2$ derived as $X^2 = n * C^2$. Only two slug species (Anisodoris nobilis and Cadlina luteomarginata) provided sufficient individuals for statistical testing at all field sites, so other species were excluded from further consideration.

Area or perimeter is probably a more biologically meaningful measure of availability to slugs as these factors determine rates of encounter more predictably than numbers of individuals (Krebs and Davies 1997). However, this approach is statistically problematic for this index for two reasons. First, combining real numbers (areas) with integers (isolations) in one ratio is improper (Sokal and Rohlf 1981). Second, Pearre's index is sensitive to differences in the relative sampling of environment versus diet (Pearre 1982). Having one set of numbers significantly larger than the other gave unrealistically low p-values (e.g., $p < 1 \times 10^{-50}$). Numbers of individual sponge patches were used instead because they are more amenable to analysis. Because number of patches scaled reasonably well with area over all the species recorded at all sites (linear regression: $n = 35$, $r^2 = 0.859$, $p < 0.0001$), the reported patterns of selection versus avoidance are similar. However, because

proportional area at each site is smaller for those sponges eaten, and larger for those sponges avoided, selectivity measures using patch number may slightly underestimate the selectivity generated using areas. Unknown sponges were lumped into one category for ease of analysis; this will not affect values for other species in such a 2 x 2 $X^2$ analysis.

Literature Review

To assess the variation in slug prey use over different geographic locations, I assembled data from published quantitative dietary studies on Northeastern Pacific Cryptobranchia. These studies are exclusively on the more common nudibranchs; data on some rarer and more specialized slugs are lacking.

**Results**

Richness and Diversity Measures

Sites differed in the number of sponge species found, with Scott's Bay (SB) having almost half again the number of sponge species of Seppings West (SW) and Dixon Reef (DXR) (Fig 4-2A). Sites also varied in species composition (Table 4-1). Rarer species were sometimes present at all sites (M. incrustans), some were present at only one site (e.g., Weberella n.sp. (Austin, unpub.)). Each site had one sponge that was dominant in both numbers and area: Plocamilla lambeii at SB and Cliona sp. at DXR and SW. Two sites also had species with a few individual patches that dominated large areas: Mycale sp at DXR and Adocia sp Reniera foraminosa at SB. Cumulative richness curves (Fig. 4-2A) suggest almost all sponge species present at

DXR were found in the survey; SB and SW may possess a few additional rare species, but the curves have nearly reached an asymptote, indicating sampling was fairly complete. Diversity also differed among sites. Cumulative diversity curves plateau sooner than richness curves (Fig. 4-2B). Again, SB showed the most diverse sponge fauna, with nearly double the diversity (H') of SW. However, DXR was not so clearly different from SB in diversity as for richness. SW curves showed a distinct pattern, reaching a plateau and then dropping with increased sampling, probably as an artifact related to the order in which the quadrats were sampled. Diversity calculated by number of patches generally paralleled diversity calculated by area, except for SW.

Prey Use and Selectivity

These slugs were selective feeders, but with a taxonomically broad prey range. Both nudibranchs used 2-4 species of prey at each site, none of which seemed to dominate their diets (Table 4-1). Approximately 10-50% of the nudibranchs captured had no spicules in their feces; this proportion was usually higher for A. nobilis than C. luteomarginata. Remnants of other organisms were often found in fecal samples, mostly barnacle exoskeletons, calcareous triaxial spicules, and diatom tests. For the demosponges sampled, slugs were highly selective for several prey items at all sites (M. incrustans, Z. hyaloderma), while others only appeared at one site (Halichondria sp., L. firma, Hymedesmia sp. A; see Fig. 4-3). Interestingly, slugs were highly selective against the most abundant sponges at each site (e.g., P. lambeii, Cliona sp.). With one exception, the degree of selectivity for these sponges did not greatly change among sites despite the different suites of prey available. L. firma's presence at DXR

is correlated with a slight reduction in use of M. incrustans by both slugs. At these three sites, there is little difference in diet composition or degree of selectivity for those items between these two slug species

**Discussion**

Richness and Diversity Measures

Field sites differed in both sponge species richness and diversity. Scott's Bay (SB) was the most species-rich, having almost half again the number of sponge species of Seppings West (SW) and Dixon Reef (DXR) (Fig. 4-2A). The factors determining these differences in species composition are unclear. Cumulative species richness curves suggest that almost all available sponge species found in survey at DXR. SB and SW almost reach an asymptote, but perhaps 5-10 additional quadrats would be required to fully assess species richness at these sites. One difference may be the "island" nature of the reef (DXR), as opposed to the somewhat arbitrarily chosen regions of subtidal that form the other sites.

These sites also differed in sponge species diversity (Fig. 4-2B). SB is the most diverse, nearly double the H' of SW. However, DXR and SB were closer in diversity than species richness, possibly because DXR lacks the large patches of Adocia spp. found at SB. These sites may present more diverse communities than in some other regions, e.g., a McMurdo Sound community, which is heavily dominated by Rosella spp. (Dayton et al. 1974). Diversity curves reached an asymptote much more quickly than richness curves, indicating that sampling was adequate to capture this descriptor. SW diversity curves reached a maximum quickly and then began dropping. This

pattern is odd, but adequately represents what was seen in the field: this site is dominated in both numbers and area by Cliona; rare species show up in early quadrats, but increased sampling obtains greater numbers of Cliona patches and decreases the observed diversity. The initial high peak in diversity was therefore likely an artifact arising from the order in which the quadrats were sampled.

Prey Use and Selectivity

Nudibranchs fed selectively, but on a taxonomically broad range of prey (Fig. 4-3). Both slugs consumed mostly poecilosclerid sponges (Table 4-1), but C. luteomarginata also consumed Halichondria spp. Surprisingly, these slugs differed little in diet composition or degree of selectivity for those items. Differences in digestive physiology between these slugs supposedly make different types of prey optimal: more highly organized skeletons, such as those in M. incrustans and Z. hyaloderma are better digested by A. nobilis than C. luteomarginata (Bloom 1976). Remains of numerous other organisms were found in fecal samples, including barnacle exoskeletons, calcareous spicules, and diatom tests. These are probably the result of incidental consumption from grazing sponges (Francis 1980) and were of insufficient quantity to suggest these slugs can survive on other classes and phyla of prey, as can Hermissenda crassicornis (Avila 1998) and Hexabranchus sanguineus (Francis 1980). The proportion of slugs lacking spicules in feces was higher for A. nobilis than for C. luteomarginata possibly because A. nobilis produces two types of feces from a given meal, one of which lacks spicules (Bloom 1976).

Slugs were highly selective for several prey items at each site (M. incrustans, Z. hyaloderma), but other prey items only appear at one site (Halichondria sp., L. firma, Hymedesmia sp. A). At all sites, nudibranchs avoided the most abundant sponge species (e.g., P. lambeii, Cliona sp.) (Fig 4-3). Even if we assume the inevitable problems with sponge identification, slugs obviously do not choose sponge prey simply by abundance. Because several of the potential problems of this study – use of OTUs for unknown sponges, problems with detecting cryptic sponge species in the field or non-spiculated sponges in slug diets – would tend to yield underestimates of diversity in both the field and in diets, slugs may actually have broader diets than described here. Degree of selectivity for particular prey doesn't greatly shift among sites despite the different sponges available, although if other acceptable prey are available, they are consumed, (e.g. L. firma at DXR). Selectivity indices are not directly comparable among sites because this index is based on different sample sizes (Pearre 1982). However, qualitative comparisons support the notion that particular sponges are always highly selected, while others are consistently avoided. Therefore, we can safely eliminate the hypothesis of random feeding with respect to local prey abundance.

Evidence from other studies

These two nudibranchs, and other species from the region, all appear to have broad diets comprised of taxonomically dissimilar prey, and rarely focus on single prey items (Table 4-2). Slug diets typically contain four to eight sponge species per site; two or three of these species usually comprise ca. 70% of the diet, and it is

unusual for > 50% of diet to be one species (e.g., Archidoris montereyensis). Both caecate and acaecate slugs appear to use sponges from numerous orders and families, indicating that skeletal architecture does not significantly restrict prey selection. One other study from Barkley Sound (Hellou et al. 1982) found a similar diet for C. luteomarginata, which is surprising given the studies collected animals from different sites and twenty years apart!

Many other dorid nudibranchs likely have similarly broad diets (Table 4-2). The Antarctic slug Austrodoris mcmurdensis consumes eight sponge species in McMurdo Sound. Most individuals (43%) ate Rosella racovitzae, but this makes up ca. 42% of sponge cover (Dayton et al. 1974). Electivity indices indicate high positive selection for four species of sponge (Gellius tenella, Haliclona dancoi, Isodictya setifera and Rosella nuda) and significant avoidance of several others, including R. racovitzae (McClintock 1987). These results are somewhat difficult to interpret, as McClintock reports values for a different suite of sponges than those in Dayton et al. (1974). Whether these merely represent taxonomic reassignments is not obvious, as he cites no taxonomic authorities. Another Antarctic slug, Austrodoris kergulensis, eats numerous genera of sponges, including some Hexactinellida (Barnes and Bullough 1996). The tropical slug Hexabranchus sanguineus is clearly a non-selective browser. Food items from only six individuals found in one study included 11 genera of sponges from 5 orders and two classes. Fecal contents also included much non-sponge material, probably from nonselective grazing (Francis 1980). The few more specialized dorids of the Northeastern Pacific (e.g., Rostanga pulchra and Aldisa cooperi) were not included in this study because too few were found. Some tropical

slugs, especially Chromodorididae, may have much more selective diets (McDonald and Nybakken 1997), but quantitative studies on most groups are lacking. Regardless, it is unclear at this point whether feeding specialization is the rule or exception for the cryptobranchs.

Geographic shifts

The dominant prey species at each site shifts geographically for the two slugs for which data exist (Table 4-2). Likewise, Austrodoris kergulensis uses different prey at different sites in Antarctica (Barnes and Bullough 1996). Two potential explanations for this shift are that potential slug diets are broad but observed diets change with prey availability, or that slug species are comprised of populations that specialize on different suites of sponges. The geographic range represented by these studies is greater than that at which genetic differentiation occurs among nudibranch populations (Todd et al. 1998), and such differentiation in prey use among populations has been seen in herbivorous sacoglossans (Trowbridge 2002). Also, little shift of prey use occurred among the populations in the current study. However, field sites in this study likely do not vary in sponge community on the same scale as would be seen across the Northeastern Pacific. Slugs often contain two or more sponge species from widely different taxa in their guts (Hellou et al. 1982, Thompson et al. 1982), so it is unclear what criteria would lead to such differentiation. Given the lack of information on sponge availability at these sites, we cannot currently eliminate either hypothesis.

**Table 4-1**. Sponge abundance and nudibranch diet data from three sites in Barkley Sound, B.C., Canada. CAMA = Cadlina luteomarginata, ANNO = Anisodoris nobilis. "None" = slugs with no identifiable spicules in their feces. Names in parenthesis after sponge names are the authority from which the names were taken; see text for details on identification. Field unknown = fecal samples for which no positive identification could be made. # = number of patches observed. * = species confirmed by Drs. W. Austin or C. Smecher.

| | Scott's Bay (SB) | | | | Seppings West (SW) | | | | Dixon Reef (DXR) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sponge species | Area ($cm^2$) | # | CAMA | ANNO | Area ($cm^2$) | # | CAMA | ANNO | Area ($cm^2$) | # | CAMA | ANNO |
| Adocia gellindra | 12 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Adocia sp. Reneira foraminosa* | 281 | 9 | 0 | 0 | 43 | 7 | 0 | 0 | 0 | 0 | 0 | 0 |
| Adocia sp | 7 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Cliona sp.* | 31 | 2 | 0 | 0 | 909 | 74 | 0 | 0 | 796 | 50 | 0 | 0 |
| Halichondria panicea* | 0 | 0 | 0 | 0 | 14 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Halichondria sp.* | 2 | 1 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Hymedesmia sp. A | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Lissodendoryx firma | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 1 | 2 | 2 |
| Mycale similar to loveni* | 49 | 1 | 0 | 0 | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Mycale sp. | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 278 | 8 | 0 | 0 |
| Myxilla incrustans | 4 | 1 | 8 | 7 | 13 | 1 | 9 | 5 | 28 | 2 | 2 | 2 |
| Pachychalina sp. | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 43 | 4 | 0 | 0 |
| Plocamilla lambeii* | 296 | 50 | 0 | 0 | 0 | 0 | 0 | 0 | 25 | 2 | 0 | 0 |
| Suberites lambeii* | 36 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Toxadocia sp. | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 55 | 2 | 0 | 0 |
| Weberella new sp. (Austin)* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 44 | 2 | 0 | 0 |
| Xestospongia vanilla* | 13 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 11 | 2 | 0 | 0 |
| Zygherpe hyaloderma | 36 | 1 | 4 | 4 | 3 | 1 | 3 | 3 | 61 | 5 | 3 | 3 |
| Diet unknown | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| Field unknown | 22 | 6 | 0 | 0 | 9 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| Number of slugs | | | 11 | 12 | | | 11 | 12 | | | 9 | 10 |
| None (empty guts) | | | 3 | 3 | | | 1 | 6 | | | 4 | 9 |
| Positive isolations | | | 15 | 11 | | | 13 | 8 | | | 8 | 7 |
| Total | | 75 | | | | 87 | | | | 78 | | |

**Table 4-2.** Quantitative studies on diets of dorid nudibranchs from the N.E. Pacific. Tabled values are percent of isolations belonging to prey sponge species. N only includes slugs with spicules in their gut; numbers of slugs examined lacking spicules in their gut were included, if known. Sponge classification from Kozloff, 1999, unless otherwise noted. Sponges are sorted by subclass (bold text) and order (listed to left). Reported diversity (H') and evenness (J) were calculated as Shannon indices. See text for details.

| Order | SLUG SPECIES / SITE | ARMO SJI | AROD SJI | DISA SJI | ANNO SJI | ANNO BMS | GEHE SJI | CAMA SCA | CAMA SJI | CAMA BMS1 | CAMA BMS2 | CAMA HS |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Tetractinomorpha** | | | | | | | | | | | |
| Axinellida | Axinella sp. | 0 | 0 | 0 | 0 | 0 | 0 | 51 | 0 | 0 | 0 | 0 |
| | Higginsia sp. | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 35 | 4 | 0 | 7 |
| | Higginsia higginissima | 0 | 0 | 0 | 0 | 0 | 0 | 9 | 0 | 0 | 0 | 0 |
| Hadromerida | Terpios sp. | 4 | 27 | 2 | 1 | 0 | 5 | 0 | 3 | 0 | 0 | 0 |
| | Suberites sp. | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 |
| | **Ceractinomorpha** | | | | | | | | | | | |
| Halichondrida | Halichondria panicea | 76 | 37 | 35 | 5 | 0 | 14 | 0 | 15 | 0 | 0 | 0 |
| | Halichondria sp. | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 8 | 0 |
| Haplosclerida | Haliclona permollis | 2 | 2 | 33 | 1 | 0 | 5 | 0 | 0 | 0 | 0 | 0 |
| Poecilosclerida | Biemna rhadia | 0 | 0 | 1 | 26 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| | Desmacella sp | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 |
| | Myxilla incrustans | 9 | 17 | 16 | 25 | 54 | 19 | 26 | 18 | 33 | 53 | 63 |
| | Lissodendoryx firma | 0 | 4 | 1 | 3 | 8 | 5 | 0 | 5 | 0 | 6 | 3 |
| | Mycale adhaerens | 4 | 5 | 8 | 14 | 0 | 19 | 0 | 5 | 0 | 0 | 0 |
| | Mycale hispida | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 |
| | Mycale lingua | 0 | 2 | 5 | 0 | 0 | 24 | 0 | 5 | 0 | 0 | 0 |
| | Mycale psila | 3 | 1 | 0 | 9 | 0 | 10 | 0 | 4 | 0 | 0 | 0 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Mycale richardsoni | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Zygherpe hyaloderma | 0 | 5 | 0 | 16 | 38 | 0 | 2 | 8 | 15 | 28 | 0 |
| Hymedesmia sp. | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 48 | 3 | 13 |
| Hymenamphiastra sp | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 |
| Forcepia sp. A | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 |
| Forcepia sp. B | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 |
| Leptolabis sp. | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 |
| # with empty gut | | | | | 18 | | 54 | | 7 | 8 | 20 |
| N | 256 | 172 | 160 | 111 | 16 | 21 | 45 | 83 | 27 | 23 | 30 |
| H' | 0.928 | 1.664 | 1.529 | 1.529 | 1.529 | 1.910 | 1.115 | 1.899 | 1.123 | 0.967 | 1.194 |
| J | 0.446 | 0.757 | 0.735 | 0.735 | 0.814 | 0.918 | 0.825 | 0.825 | 0.810 | 0.601 | 0.667 |
| Source | B | B | B | B | BKP | B | T | B | H | BKP | H |

**Sources**

| | |
|---|---|
| B | Bloom 1974 |
| H | Hellou et al. 1982 |
| T | Thompson et al. 1982 |
| BKP | current study |

**Slugs**

| | |
|---|---|
| ARMO | Archidoris montereyensis |
| AROD | Archidoris odhneri |
| DISA | Diaulula sandiegensis |
| ANNO | Anisodoris nobilis |
| GEHE | Geitodoris heathii |
| CAMA | Cadlina luteomarginata |

**Site**

| | |
|---|---|
| SJI | San Juan Island |
| SCA | Southern California |
| BMS | Bamfield Marine Station, B.C. |
| 1 | Hellou et al. 1982 |
| 2 | This study |
| HS | Howe Sound, B.C. |

**Figure 4-1.** Map of field sites for diet study. DXR = Dixon Reef, SB = Scott's Bay, SW = Seppings Island, West. North is to the top of the map.

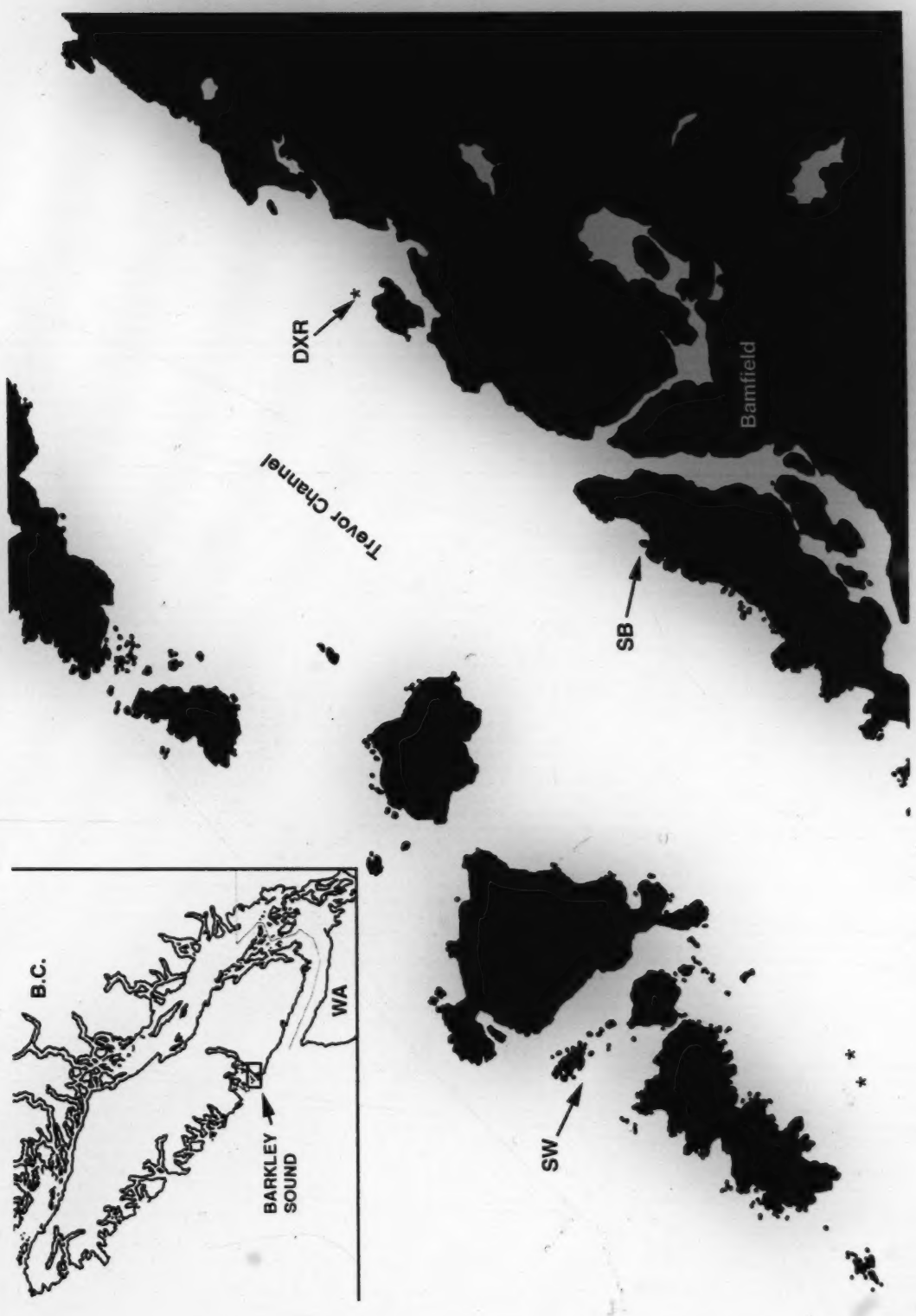
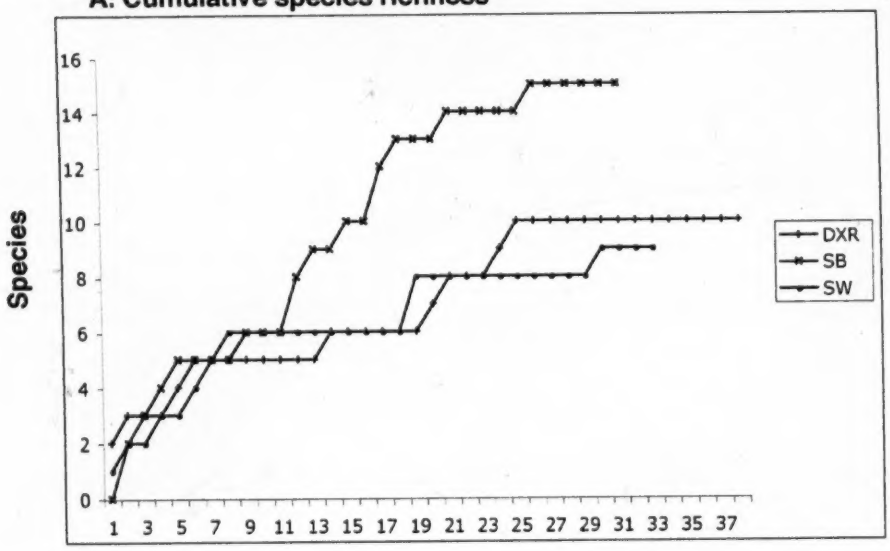

DXR
Bamfield
Trevor Channel
SB
SW
B.C.
WA
BARKLEY SOUND

**Figure 4-2.** Sponge community sampling at three subtidal sites in Trevor Channel, B.C. DXR = Dixon Reef, SB = Scott's Bay, SW = Seppings West. A. Cumulative species richness by quadrat number, in order of sampling. B. Cumulative species diversity, determined both as number of sponge patches and total area of sponge.

**A. Cumulative species richness**




**B. Cumulative diversity**

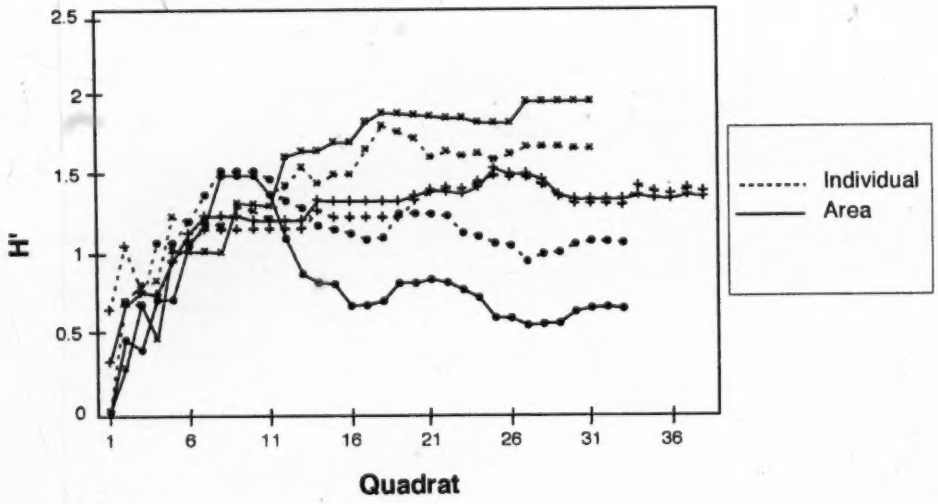
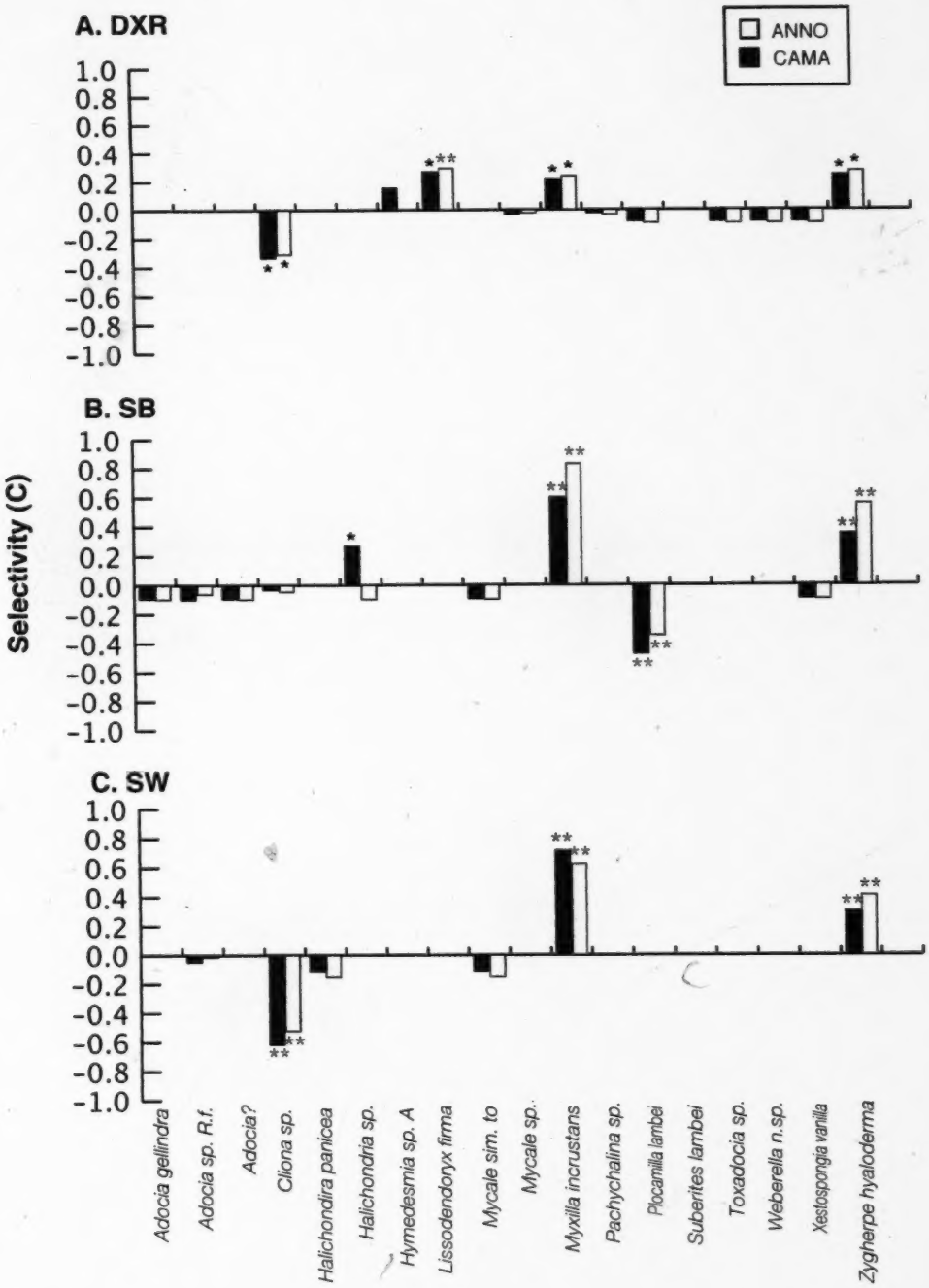


**Figure 4-3.** Nudibranch feeding selectivity at three sites in Trevor Channel, B.C., measured as Pearre's index (C). A. Dixon Reef. B. Scott's Bay. C. Seppings West. Positive values indicate selection for a prey item, negative values indicate avoidance. * = $p < 0.05$, ** = $p < 0.01$.


A. DXR
ANNO
CAMA
B. SB
C. SW
Selectivity (C)
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
Adocia gellindra
Adocia sp. R.f.
Adocia?
Cliona sp.
Halichondira panicea
Halichondria sp.
Hymedesmia sp. A
Lissodendoryx firma
Mycale sim. to
Mycale sp.
Myxilla incrustans
Pachychalina sp.
Plocamilla lambei
Suberites lambei
Toxadocia sp.
Weberella n.sp.
Xestospongia vanilla
Zygherpe hyalioderma

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# CHAPTER 5

# PHYLOGENETIC VALUE OF SPICULE NETWORKS IN DORID NUDIBRANCHS

## Introduction

Dorid nudibranchs (Gastropoda: Opisthobranchia) are a key taxon for understanding the evolution of chemical defense (Cimino et al. 1999) and feeding specialization (Karuso 1987) in the marine environment, yet a comprehensive and accurate phylogeny remains elusive. Gastropods as a whole are problematic taxonomically (Ponder and Lindberg 1996), and nudibranchs particularly because of their lack of a shell, which provides numerous phylogenetic characters for other gastropod groups (Foale and Willan 1987). This, coupled with a high level of parallelism (Gosliner 1985), has impeded agreement on a proper phylogeny. Future advances in nudibranch systematics will depend on the discovery of new, phylogenetically informative characters.

Many cryptobranchiate dorids possess calcareous spicules (Hyman 1967). These spicules often comprise a large portion of the animal's dry weight (Cattaneo-Vietti et al. 1993, Chapter 3), to the degree that these slugs almost hold their shape when dried, unlike unspiculated nudibranchs (B. Penney, personal observation). Spicule form and presence have been investigated as potential phylogenetic characters, and have been useful for resolving some groups (Valdés and Gosliner 1999), but not others (Valdés and Gosliner 2001). However, spicules often occur in

discrete networks (Garcia et al. 1986, Chapter 3) with some variation between the two species investigated (Doriopsilla areolata and Cadlina luteomarginata, respectively) (Chapter 3). These networks may provide useful phylogenetic information, but we do not currently know their form in enough taxa (Wägele and Willan 2000).

I therefore documented spicule network form in 15 taxa of dorid nudibranchs to test whether variation among taxa was clear and consistent enough for use as a phylogenetic character. To this end, I contrasted network form within two taxa of cryptobranchiate dorids known to be monophyletic – the Porostomata (Valdés and Gosliner 1999) and the caryophyllidia-bearing dorids (Valdés and Gosliner 2001) – to ask whether this character adequately distinguished them. To assess the polarity of these new characters I also investigated several common outgroup species. Lastly, I tested these characters on several species common in the Northeastern Pacific with poorly-known taxonomic affinities.

## Methods

### Taxa and Relationships

This study covered 16 opisthobranch species, representing a range of outgroups, known cryptobranchiate taxa, and species with uncertain affinities (Table 5-1; Fig. 5-16). The Doridacea are probably monophyletic with the Pleurobranchoidea as their sister group (Wägele and Willan 2000), so the common Berthella californica was included as an outgroup. Although pleurobranchs still retain a thin internal shell, they also possess star-shaped calcareous spicules (Hyman 1967). Within the Doridacea, Bathydoris has previously been used as an outgroup (Valdés

and Gosliner 1999, Wägele and Willan 2000, Valdés and Gosliner 2001) because of its primitive features. More highly-derived dorids are typically split into the Phanerobranchia and the Cryptobranchia, the latter having a gill ring that can be withdrawn into a pocket (Schmekel 1985). From the phanerobranchs, I selected Acanthodoris nanaimoensis, a common slug that is superficially similar to cryptobranchs in morphology.

Within the Cryptobranchia, the genus Hexabranchus is usually considered basal (Wägele and Willan 2000). The Chromodorididae likely split off next, with Cadlina as a basal genus to the family (Rudman 1984, Wägele and Willan 2000). Placement of other taxa is unclear, but two monophyletic groups have been identified. The caryophyllidia-bearing dorids possess specialized sensory tubercles on their notum, with the mantle cilia found exclusively on these projections (Valdés and Gosliner 2001). This unnamed taxon contains 3-4 groups of genera whose relationships to each other are not resolved. Diaulula, Sclerodoris and Atagema each represent one of these subgroups. The Porostomata Bergh 1878 are distinguished by lack of a radula and salivary glands, possesion of oral glands and other features (Valdés and Gosliner 1999). The Porostomata is further split into the newly described basal genus Mandelia, plus two major branches: the Phyllidia/Phyllidiopsis group and the Dendrodoris/Doriopsilla group.

Anisodoris nobilis, Archidoris montereyensis and Aldisa sanguinea are common Northeastern Pacific cryptobranchs previously used in studies of chemical defense (Avila 1995) and feeding specialization (Bloom 1976). All have a labium on the anterior foot and multipinnate gills, making them more highly-derived than the

Chromodorididae (Valdés and Gosliner 1999). The genus Anisodoris is no longer valid and requires revision (Valdés and Gosliner 2001). However, because A. nobilis has a notched bilabiate foot and digitiform oral tentacles (MacFarland 1966), it is probably closely allied with the caryophyllidia-bearers (T. Gosliner, personal communication). The genus Archidoris has not been recently reviewed, but A. pseudoargus has appeared between Cadlina and higher taxa in several studies of cryptobranch phylogeny (Valdés and Gosliner 2001) (Wägele and Willan 2000). Aldisa has previously been placed with Rostanga based on radular morphology and color (Millen and Gosliner 1985), but morphological characters such as notal structure, oral tentacles, and labial form contradict this placement.

## Specimens

Specimens were obtained either from the California Academy of Sciences collection, or by SCUBA from Barkley Sound B.C. New specimens were preserved in buffered formalin for 1-3 d before transfer to 95% ethanol (Table 5-1). For museum specimens, I chose specimen lots that contained several specimens of different sizes, and that had not been preserved in unbuffered formalin, as the acidic nature of this fluid will dissolve spicules (G. Williams, personal communication). One to two individuals of each species were chosen for staining, based on state of contraction and overall condition.

Staining

Following the methods given in Chapter 3, specimens were rinsed briefly in 95% ethanol to remove remaining preservative, then larger specimens were dissected into foot, mantle, rhinophores and gills to allow better visualization. Viscera were not further treated, but were kept as vouchers. Samples were cleared in phenol over 2 d until translucent, then further digested for 1-3 d in a stock alcohol-hydroxide solution (70% EtOH and 10% NaOH, mixed 15:1). Cleared samples were then stained with Alizarin red mixed at 0.03% by weight in the above alcohol hydroxide solution. Overstaining of the body tissues was removed through overnight treatment in a 1:1 mixture of the stock alcohol-hydroxide solution to glycerol, which did not seem to affect the staining of calcareous elements. Samples were then mounted in glycerol to further clear tissues. For large-bodied specimens for which this method did not allow adequate visualization, I hand cut cross-sections of tissues to approximately 1 mm thickness.

These procedures stain calcium carbonate a deep red and render other tissue nearly colorless and translucent. Therefore, spicules appear as dark bodies in almost all photos, except where noted. Tissues were photographed through a Wild Stereomicroscope fitted with a Nikon DMX 1200 digital camera, and images captured using Nikon "Act1" software. One figure (Fig. 5-13C) is a montage of two pictures taken with different focus, which were precisely aligned and flattened into a single image using Adobe Photoshop™ to create a picture with a better depth of field.

## Results

Characters

Several aspects of spicule networks were distinct enough and sufficiently variable for use as phylogenetic characters. More characters were based on the mantle, as mantle structures were often more distinct among taxa, and were also easier to photograph clearly. Rhinophore and gill characters may vary enough to provide phylogenetic information (Chapter 3), but were not visible in many specimens that were preserved in a contracted state and were therefore excluded. For taxa lacking spicules, all remaining characters were coded as undefined. Rather than a formal phylogenetic analysis, character states for each species were mapped onto a phylogeny based on recent literature (Valdés and Gosliner 1999, 2000; Wägele and Willan 2000), with affinities for A. nobilis, A. montereyensis and A. sanguinea resolved using spicule characters (Fig. 5-16).

*1. Spicule presence.*

(0) Spicules present.

(1) Spicules absent.

*2. Mantle network form.*

(0) Spicules generally solitary and arranged haphazardly, not arranged into any obvious network (Fig. 5-2A).

(1) A dendritic, ramifying network of multispicular tracts, with some radial tracts evident but not dominating (Fig. 5-1A; 5-10A).

(2) A cobweb-like form, similar to an isodictyal sponge skeleton (Fig. 5-1B; 5-5A,B).

(3) A highly organized, lattice-like network, with clear radial and circumferential tracts (Fig. 5-1C, 5-12A).

*3. Central notum network.*

(0) Network in the notum center is the same as in the mantle edge (Fig. 5-3A).

(1) Spicules rare or absent in the central notum.

(2) The central notum contains comparatively few spicules, which are significantly larger than those in the mantle edge (Fig. 5-12A).

*4. Papillae support.*

(0) Notal structures not supported by spicules (Fig. 5-3D).

(1) Papillae supported by vertical tracts of spicules that end in a disorganized plush of spicules (Fig. 5-1D; 5-10D).

(2) Notal structures supported by a distinct ring of spicules (Fig. 5-1E; 5-5C), whether or not the soft tissue is organized to form caryophyllidia.

(3) Papillae supported by rods of few large spicules (Fig. 5-12C).

*5. Foot network form.*

(0) No obvious organization or network (Fig. 5-2B).

(1) Dominated by conspicuous multispicular tracts (Fig. 5-1A; 5-11E).

(2) A cobweb form, as in character 3 (Fig. 5-1B; 5-13D).

Outgroups

*Berthella californica*

Stellate spicules were arranged haphazardly in both foot (Fig. 5-2A) and mantle regions near the body wall (Fig. 5-2B). Spicules did not seem to continue throughout the entire mantle, and were instead replaced by a network of conective tissue (not shown). Spicules in the border of both the foot and oral veil were arranged more tightly, but not in more conspicuous order (Fig. 5-2D). All spicules were stellate.

*Bathydoris aioca*

B. aioca did not possess any spicules (not shown). The mantle was very thin, similar to that of prosobranchs. Foot tissue did not clear as well as that of other groups in this study, despite similar treatment; this may suggest differences in foot structure compared to other opisthobranchs.

*Acanthodoris nanaimoensis*

The mantle was thick and covered with papillae, superficially like cryptobranchiate dorids. The mantle network was comprised of multispicular tracts (Fig. 5-3A). These tracts were still distinct, but became much less dense as they crossed the central notum (Fig. 5-3B), while the mantle edge was densely packed with tracts and loosely aligned cross-braces of one to several spicules (Fig. 5-3C). Vertical tracts extended up from the main network, but were only aligned with a few papillae and did not extend beyond the bases (Fig. 5-3D). The organization in the foot was ambiguous, but closer to a cobweb-like arrangment than multispicular tracts (Fig. 5-4A). Spicules were almost absent from the center of the foot (Fig. 5-4B), but were more heavily packed as they approached the foot edge (Fig. 5-4C).

*Hexabranchus sanguineus*

Neither mantle nor foot contained spicules (not shown).

Caryophyllidia-bearing dorids

*Diaulula sandiegensis*

The mantle contained a thin, cobweb-like network of spicules loosely arranged into an unbraced framework, usually with only single spicules making up the sides of the framework (Fig. 5-5A,B). This network continued through the central region of the notum. Caryophyllidia projected from the mantle surface, with numerous spicules supporting a sensory bulb and spicules integrated into the interior network just under the mantle surface (Fig. 5-5C,D). The foot showed a fine cobweb

arrangement similar to the mantle (Fig. 5-6A, B). This network was continuous across the width of the foot, although less dense in the center (Fig. 5-6A). The foot border was densely packed with fine tracts (Fig. 5-6C), the ends of which had a distinct arrangement (Fig. 5-6D). There were no distinct differences between the two specimens examined.

*Atagema alba*

The mantle had a cobweb-like arrangement similar to Diaulula (Fig. 5-7A, B), with small caryophyllidia integrated into the main spicule network just under the mantle surface (Fig. 5-7C). The network continued throughout the central notum. The oral tentacles had a mesh of spicules running down the center (Fig. 5-7D). The foot had a more heavily-spiculated border than the mantle, with discrete structures at ends of tracts (Fig. 5-7E). It was not possible to examine the middle of the foot, as the small size of the specimen precluded dissection.

*Sclerodrodis coriacea*

The mantle contained a dense cobweb network, similar to Diaulula and Atagema (Fig. 5-8A,B,D). There were rings of spicules supporting small caryophyllidia on the surface, again integrated into the spicule network just under the mantle surface (Fig. 5-8C). The foot also contained a cobweb-like network, more open than in the mantle (Fig. 5-9A). This network became less dense near the foot midline (Fig. 5-9B). Spicules were almost organized into tracts in some places (Fig.

5-9C). The foot border contained a very dense, disorganized mesh, almost like the mantle in places (Fig. 5-9D).

Porostomata

*Doriopsilla albopunctata*

The mantle edge contained a ramifying mesh of distinct multispicular tracts (Fig. 5-10A), whereas the central notum contained only large individual spicules (Fig. 5-10A,B). The outer edges of the mantle showed tracts of decreasing width, fading out with no definite border (Fig. 5-10C). Papillae rose from the main network on short vertical multispicular tracts, ending in a disorganized plush of spicules (Fig. 5-10D). The foot edge had a ramifying network, with horizontal tracts through the central region (body wall to body wall) that were not noticeably less dense in the center (Fig. 5-10E).

*Dendrodoris albobrunnea*

Neither the foot nor the mantle contained any spicules (not shown), despite similarity in form to other dorids.

*Phyllidia varicosa*

The mantle edge showed a ramifying network of multispicular tracts, with radial tracts nearly parallel, and extending almost perpendicular to the body. These radial tracts met the thinner circumferential tracts at almost a 90° angle (Fig. 5-11A). Both types of tracts became narrower, more finely divided and less distinct from each

other near the mantle edge (Fig. 5-11B). There was no distinct border to the network around the mantle edge. Papillae were supported by vertically rising tracts of small spicules; these tracts spread out into a large plush of increasingly fine tracts near the papilla surface (Fig. 5-11C). The central notum contained a diffuse mesh of overlapping spicules that were several orders of magnitude larger than the spicules in the mantle edge (Fig. 5-11D). The foot contained loose disorganized tracts of large spicules, some of which form multispicular horizontal tracts (Fig. 5-11E).

*Phyllidiopsis cardinalis*

The mantle edge contained wide multispicular tracts, with radial tracts noticeably wider than circumferential tracts (Fig. 5-12A). Radial tracts were nearly parallel to each other, and circumferential tracts were somewhat perpendicular to them. Tracts became somewhat more finely divided as they approached the mantle edge; there was no distinct spicule border at the mantle edge (Fig. 5-12B). Papillae were supported by rods of a few large spicules; these did not branch into plushes (Fig. 5-12C). The central notum contained numerous large spicules, not organized into tracts but lying at approximately 45° to either side of the body axis (Fig. 5-12D). The foot contained a disorganized mesh of single, intermediately-sized spicules, with an area in the center of the foot that lacked spicules altogether (Fig. 5-12E).

Other Cryptobranchia

*Archidoris montereyensis*

The mantle contained a densely packed mesh of mostly single spicules that formed an unbraced framework that continued through the central notum (Fig. 5-13A,B). Short vertical tracts extended into the base of papillae, where they met with cross-braces and eventually erupted into a disorganized plush of spicules (Fig. 5-13C). The foot had a network similar to the mantle (Fig. 5-13D), with no definite border at the edge (Fig. 5-13E). The central foot was too thick to see the relative degree of spiculation, but its pattern was similar to that of the foot edge. There were no noticeable differences between the two specimens studied.

*Anisodoris nobilis*

The mantle contained a dense, cobweb-like mesh of spicules without obvious tracts, and in which spicules seemed to form clusters at irregular intervals (Fig. 5-14A). The central notum lacked a network of spicules under the papillae (not shown). Papillae were supported by a regular ring of spicules (Fig. 5-14B). The foot had a less-dense irregular mesh of single spicules that faded toward the center (Fig. 5-14C). The anterior lip of the foot (labium) had a very regular set of structures at its border (Fig. 5-14D). There were no noticeable differences between the two specimens studied.

*Aldisa sanguinea*

The mantle had conspicuous multispicular tracts leading toward the mantle edge, connected by cross-braces at irregular intervals (Fig. 5-15A). These were not as consistent as the radial and circumferential tracts found in other taxa. Papillae were supported by a disorganized plush of spicules rising from short vertical tracts coming out of the basal network (Fig. 5-15B). Radial tracts only became slightly thinner as they approached the mantle edge (Fig. 5-15C). The central notum was almost bare of spicule network, but contained a few larger spicules near the edges (Fig. 5-15A). The foot contained a less-organized network of multispicular tracts, with the central region of the foot having only a few large spicules (Fig. 5-15D). Tracts became somewhat thinner toward the edge of the foot, but there was no definite border (Fig. 5-15E). There were no noticeable differences between the two specimens studied.

**Discussion**

Spicule network characters were generally consistent in the two known groups of cryptobranchs. All three caryophyllidia-bearing dorids examined had a similar cobweb-like arangement of spicules (Fig. 5-1B) throughout the mantle and at least parts of the foot (Figs. 5-5 through 5-9), though the density of spicules varied. All papillae were supported by a distinct ring of spicules, and the central notum had the same type of network as the edge. Rostanga pulchra has a similar pattern of spiculation (Chia and Koss 1978, Penney and Koss unpublished) as does Taringa timia (B. Penney, unpublished). Interestingly, Diaulula sandiegensis and Atagema alba looked more similiar to each other than either did to Sclerodoris coriacea, as the

latter shows heavier spiculation in the mantle, more discrete tracts in the foot, and a less-organized border to the foot. The larger groups to which these species belong are not well-resolved (Valdés and Gosliner 2001), so further investigation of spicule networks may better clarify relationships within caryophyllid-bearing dorids.

Within the Porostomata variation was somewhat greater (Figs. 5-10, 5-11). Only Dendrodoris lacked spicules, confirming previous studies (Valdés and Gosliner 1999). Phyllidia varicosa and Phyllidiopsis cardinalis had very similar patterns of mantle spiculation, with obvious radial tracts connected by organized circumferential tracts. The central notum had large spicules at approxitely 45° to the main body axis. Their form of papillae support differed, however (Figs. 5-11C, 5-12D); this may prove a useful character for lower levels of resolution. Doriopsilla albopunctata also shared this pattern, but this differed from D. areolata (Garcia et al. 1986), which has a disorganized mesh of spicules in both the mantle edge and center. D. areolata belongs to a crown group within the genus, and is closely related to D. albopunctata, so it is unclear which state is more derived for this genus.

Outgroups both within and outside the Cryptobranchia showed a great deal of variation in spicular characters. Both Cadlina (Chapter 3) and Acanthodoris nanaimoensis (Fig. 5-3) had disorganized meshes of multispicular tracts, although these were more clearly organized in Cadlina. Berthella californica (Fig. 5-2) had a different form of spicule that was arranged without apparent organization, while Bathydoris aioca lacked spicules. Calcareous spicules may therefore be independently derived for the dorid nudibranchs. However, Hexabranchus sanguineus also lacks spicules. Coupled with the lack of spicules in more-derived taxa such as the

Chromodorididae and Dendrodoris, spicules probably have been lost multiple times within the dorids (Valdés and Gosliner 1999). Therefore, the most likely ancestral state for the cryptobranch dorids is a network consisting of a ramifying network of disorganized multispicular tracts (Character 2, State 1), with vertical tracts of spicules rising into a disorganized plush of spicules supporting papillae (Character 4, State 1), and a similar pattern of spiculation in both the mantle edge and center (Character 3, State 0; Fig. 5-16). The ancestral pattern of foot spiculation is less clear because no two basal taxa share a similar pattern.

Spicule networks appear to provide clearer affinities for the ambiguous groups studied, especially when combined with other morphological characters (Fig. 5-16). Anisodoris nobilis shares with the caryophyllidia-bearing dorids a cobweb-like arrangement of mantle spicules and papillae supported by a distinct ring of spicules (Fig. 5-14), as well as a notched bilabiate foot and digitiform oral tentacles (MacFarland 1966). However, because it lacks the same sensory bulb arrangement found in caryophyllidia and a spicule network in the central notum, it cannot be placed within this group. Archidoris montereyensis also has a cobweb-like arrangement of mantle spicules (Fig. 5-12), but its papillae with disorganized plushes of spicules, un-notched labium and flaplike oral tentacles (MacFarland 1966) suggest a more basal position on this branch (Fig. 5-16). Aldisa sanguinea has a disorganized mesh of spicule tracts throughout its mantle (Fig. 5-15), but the presence of only a few larger spicules in the mantle center, plus reduced flap-like oral tentacles (Millen and Gosliner 1985) may suggest a relationship with the Porostomata. Although this is

more tenuous that the relationships proposed for A. nobilis and A. montereyensis, I have no characters to refute it and have therefore used this topology in Fig. 5-16.

Generally, this method of investigating spicule networks may provide useful new characters for investigating the systematics of the Doridacea. It is quick, easy, and provides relatively distinct characters. While some aspects of spicule networks, such as in the foot, may be variable within taxa, some characters seem quite reliable. Additionally, characters seemed invariable within species among the 2-4 specimens examined for most species, unlike some aspects of brain morphology (Valdés and Gosliner 2001). A reliable phylogeny for this group would greatly facilitate evolutionary studies of chemical defense, feeding specialization, and other characters.

**Table 5-1.** Species examined for staining of spicule networks. I used confirmed monophyletic groups for comparision, but because some of these have not been officially named (Valdés and Gosliner 2001) some of the following "Group" names are simply descriptive. *, specimens collected from Bamfield Marine Station, **, data taken from the literature. For details of classification, see Table 1-1.

| Group | Species | Number and Size |
|---|---|---|
| Pleurobranchoidea | *Berthella californica | 1; 3.9 cm |
| Doridoxoidea | Bathydoris aioca | 1; 2.4 cm |
| Phanerobranchia | *Acanthodoris nanaimoensis | 2; 2.1, 2.8 cm |
| Doridacea | Hexabranchus sanguineus | 1; 2.9 cm |
| Cryptobranchia | **Cadlina luteomarginata | (Chapter 3) |
| Caryophyllidia | *Diaulula sandiegensis | 2; 3.2, 5.4 cm |
| Caryophyllidia | Atagema alba | 1; 0.9 cm |
| Caryophyllidia | Sclerodoris coriacea | 1; 2.6 cm |
| Porostomata | **Doriopsilla areolata | (Garcia et al. 1986) |
| Porostomata | Doriopsilla albopunctata | 1; 1.9 cm |
| Porostomata | Dendrodoris albobrunnea | 1; 3.1 cm |
| Porostomata | Phyllidia varicosa | 1; 1.9 cm |
| Porostomata | Phyllidiopsis cardinalis | 1; 2.0 cm |
| Cryptobranchia | *Archidoris montereyensis | 2; 3.1, 4.8 cm |
| Cryptobranchia | Anisodoris nobilis | 2; 1.7, 2.8 cm |
| Cryptobranchia | Aldisa sanguinea | 2; 0.9, 1.4 cm |

**Figure 5-1.** Schematic drawings of typical spicule networks of cryptobranchiate dorids. Hatching density indicates density of spicules in each region for A and B; outlines indicate individual spicules in C and E. S = surface of notum.

A. Ramifying dendritic network, modeled after C. luteomarginata. Bar = 0.5 mm.

B. Cobweb network, modeled after A. montereyensis. Bar = 2 mm.

C. Lattice-like network with circumferential and radial tracts, modeled after P. varicosa. Bar = 1 mm.

D. Vertical tracts of spicules, leading to a surface plush that supports the papilla. Based on C. luteomarginata. Spicules are shown as dark masses. Dotted line = approximate limit of connective tissue sheath. Bar = 0.5 mm.

E. Ring of spicules supporting caryophyllidium, with underlying spicule network. Based on D. sandiegensis. Dotted line indicates approximate limit of soft tissue on surface of caryophyllidium. Bar = 0.3 mm.

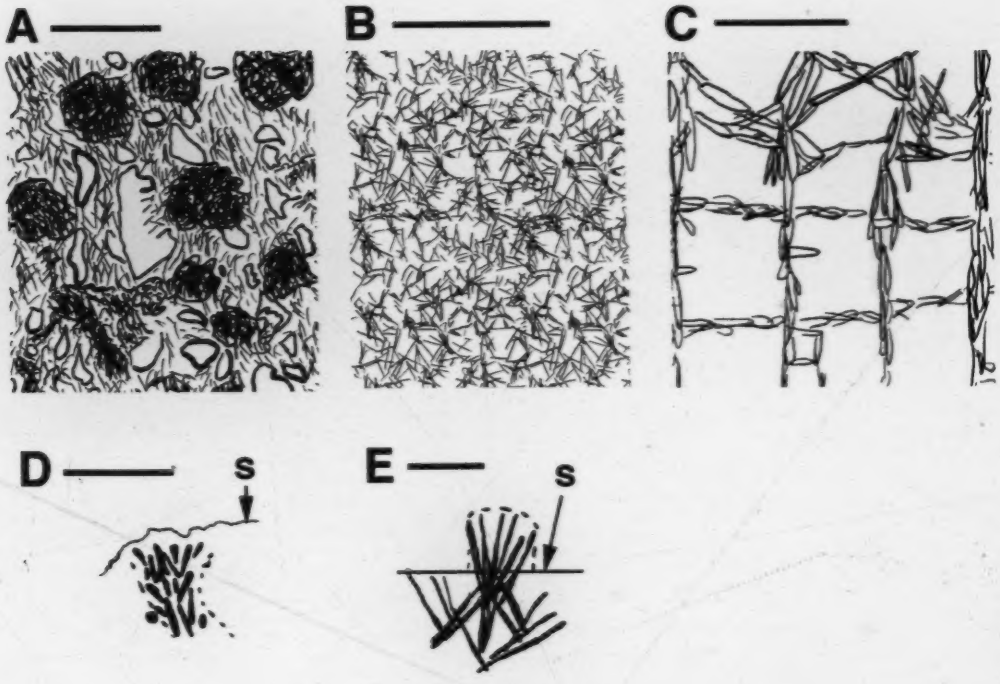

A
B
C
D
s
E
s

**Figure 5-2.** Spicule networks of Berthella californica. The anterior side of the specimen is to the top in all panels, except C.

A. Dorsal view of cut edge of body wall between foot and mantle (foot side), to show spicule arrangement. Exterior edge of the foot is to the right. Stained spicules show to the right, with a region of unstained spicules in the lower left. Bar = 0.5 mm.

B. Ventral view of cut edge of body wall between foot and mantle (mantle side). Bar = 2.5 mm.

C. Ventral view of the foot border, with the anterior of the animal to the right. Bar = 1 mm.

D. Higher magnification of the area immediately left of center in B, to show spicule shape. Bar = 1 mm.

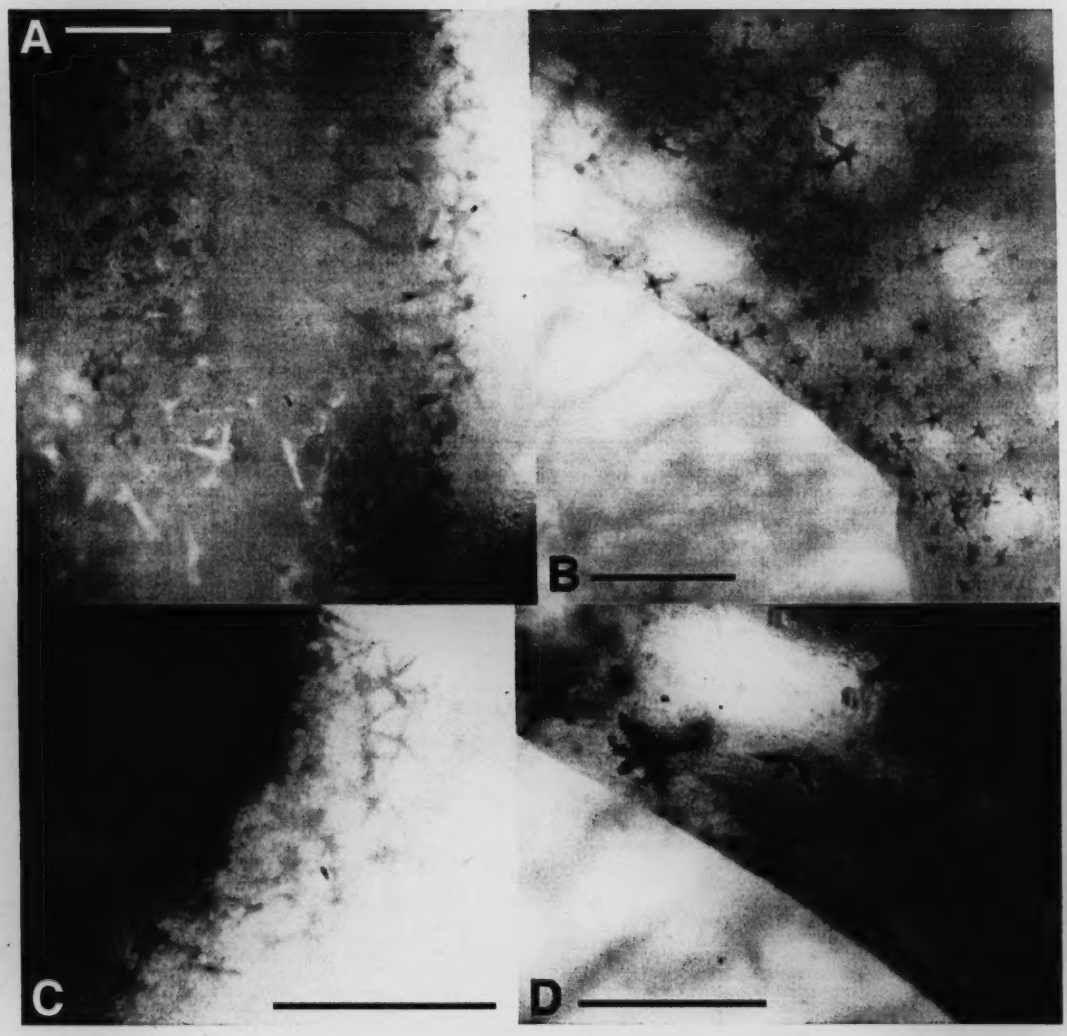

A
B
C
D

**Figure 5-3.** Spicule networks of Acanthodoris nanaimoensis mantle. The anterior side of the specimen is to the top in all panels, except D.

A. Ventral view of the anterior right quarter of mantle. Bar = 2.5 mm.

B. Higher magnification of the area just right of the top center in A. Bar = 2.5 mm.

C. Higher magnification of the mantle edge in the bottom left of A. Bar = 0.5 mm.

D. Side view of a mantle cross-section, with the dorsal surface at the top of the image. Bar = 5 mm.

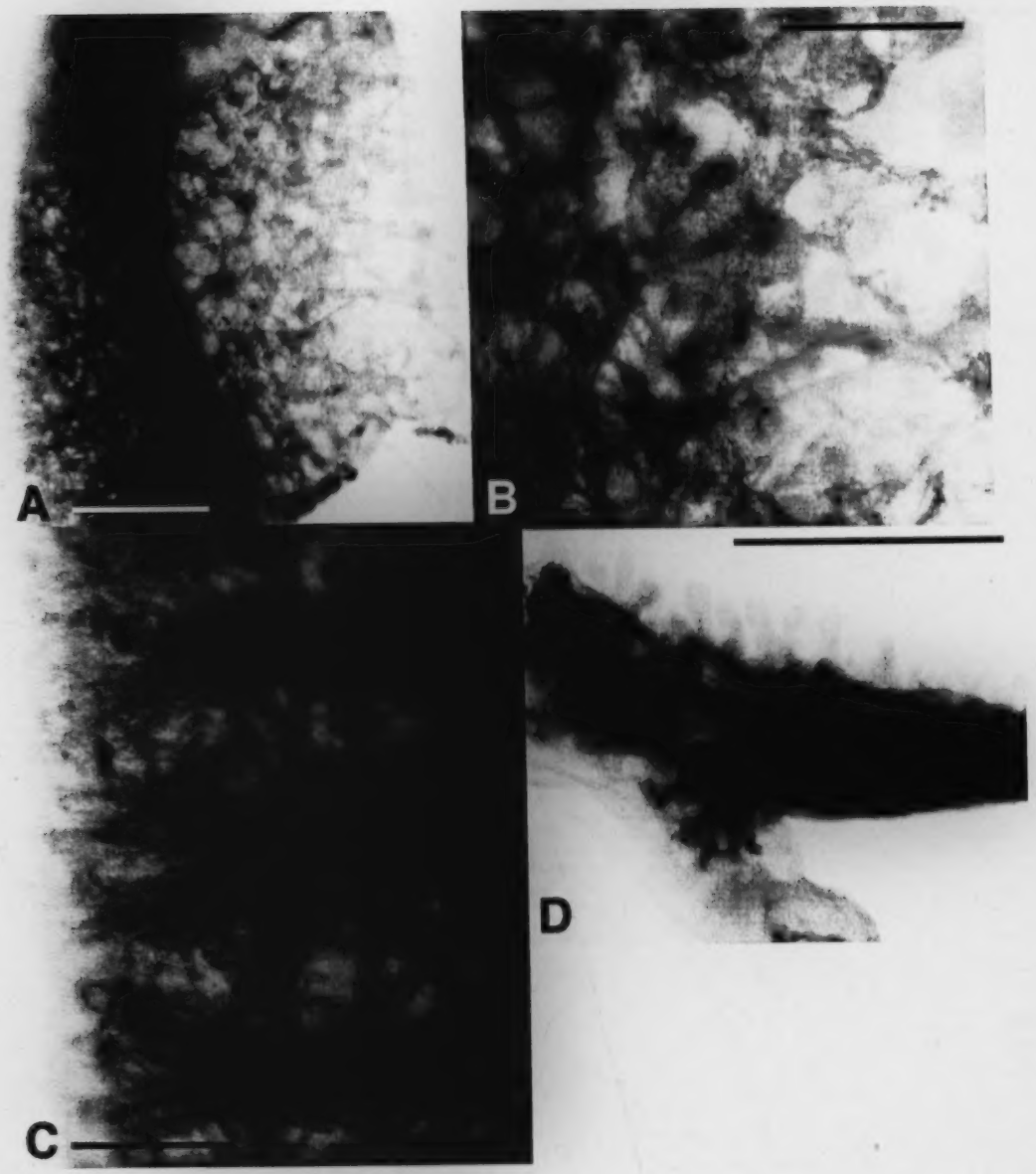

A
B
C
D

**Figure 5-4.** Spicule networks of Acanthodoris nanaimoensis foot. The anterior side of the specimen is to the top in all panels.

A. Ventral view of the posterior foot. Bar = 2.5 mm.

B. Higher magnification of the central region of A. Bar = 1.5 mm.

C. Higher magnification of the posterior edge of foot in A. Bar = 1.5 mm.

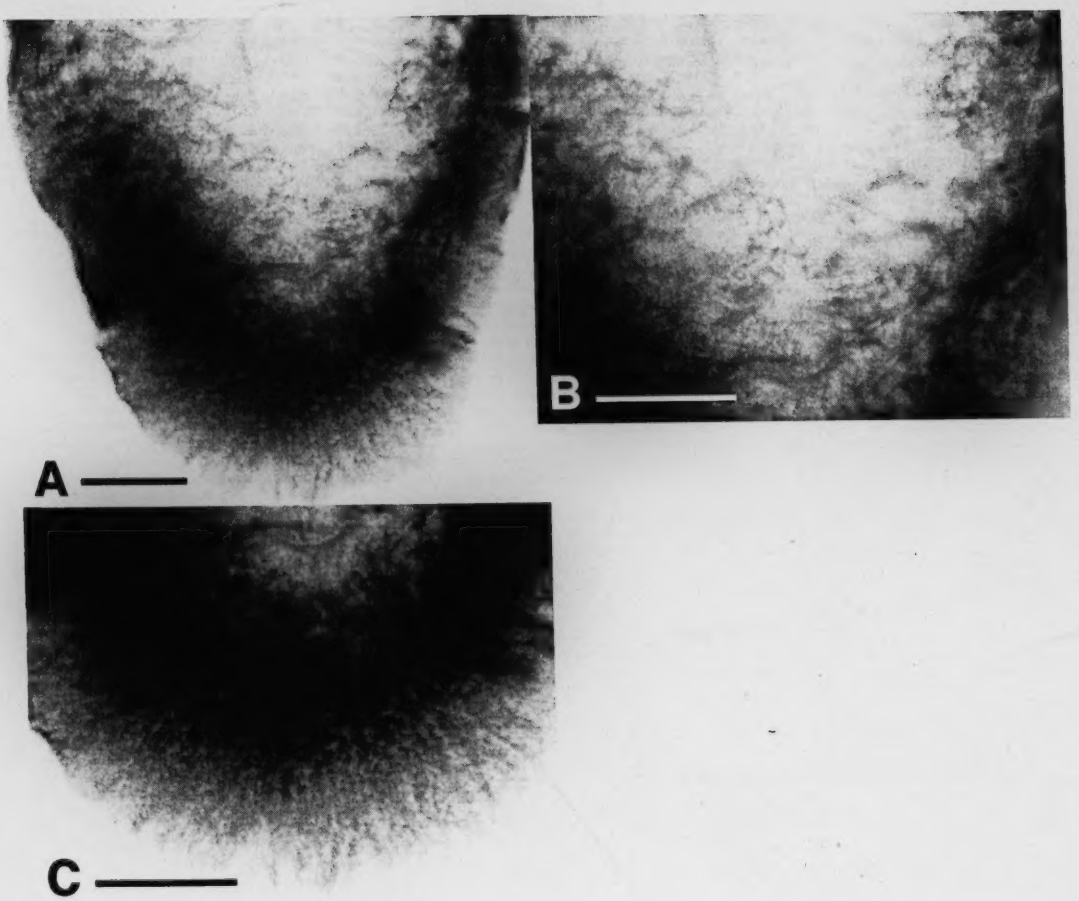

A
B
C

**Figure 5-5.** Spicule networks of Diaulula sandiegensis mantle. The dorsal surface of the specimen is to the top in all panels except A.

A. Inverted-color image of a saggital section of mantle near the body wall; the spicule networks appear as white. Bar = 2 mm.

B. Cross section of the mantle, near midline of body. Bar = 2 mm.

C. Cross section of mantle, near the surface, to show the integration of caryophyllidia with underlying network (arrow). The cut face of the mantle occupies approximately the bottom half of the image. Bar = 1 mm.

D. Mantle surface to show caryophyllidia and underlying surface network. Bar = 1 mm.

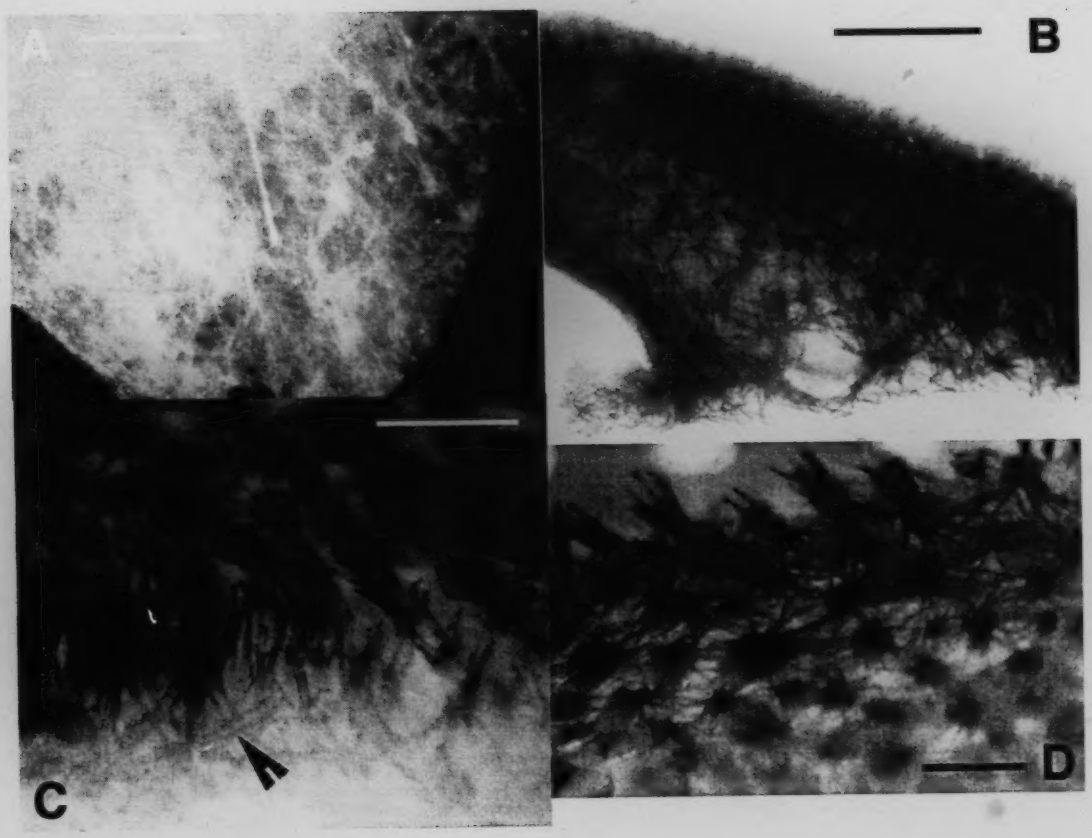

A
B
C
D

**Figure 5-6.** Spicule networks of Diaulula sandiegensis foot. The anterior side of the specimen is to the top in all panels.

A. Ventral view of the foot near the posterior end. Bar = 2.5 mm.

B. Enlarged ventral view of the midline region of foot, near the middle of the body. Bar = 0.5 mm.

C. Higher magnification of the foot edge from the bottom center of A show the cobweb-like network of spicules. Bar = 1 mm.

D. Higher magnification of the foot edge from the top left of C. Bar = 0.4 mm.

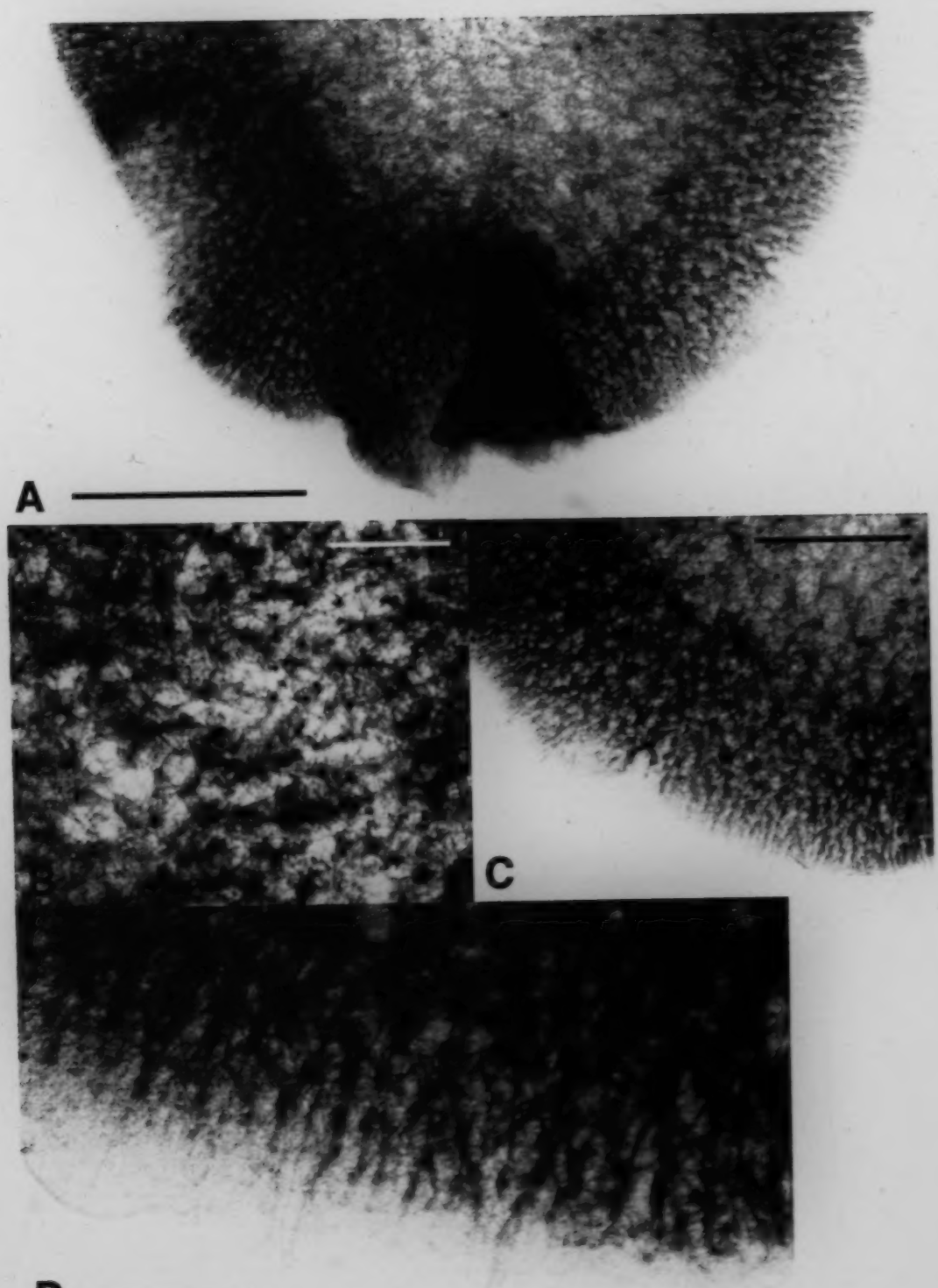

A
C
D

**Figure 5-7.** Spicule networks of Atagema alba. The anterior side of the specimen is to the right in all panels, except D.

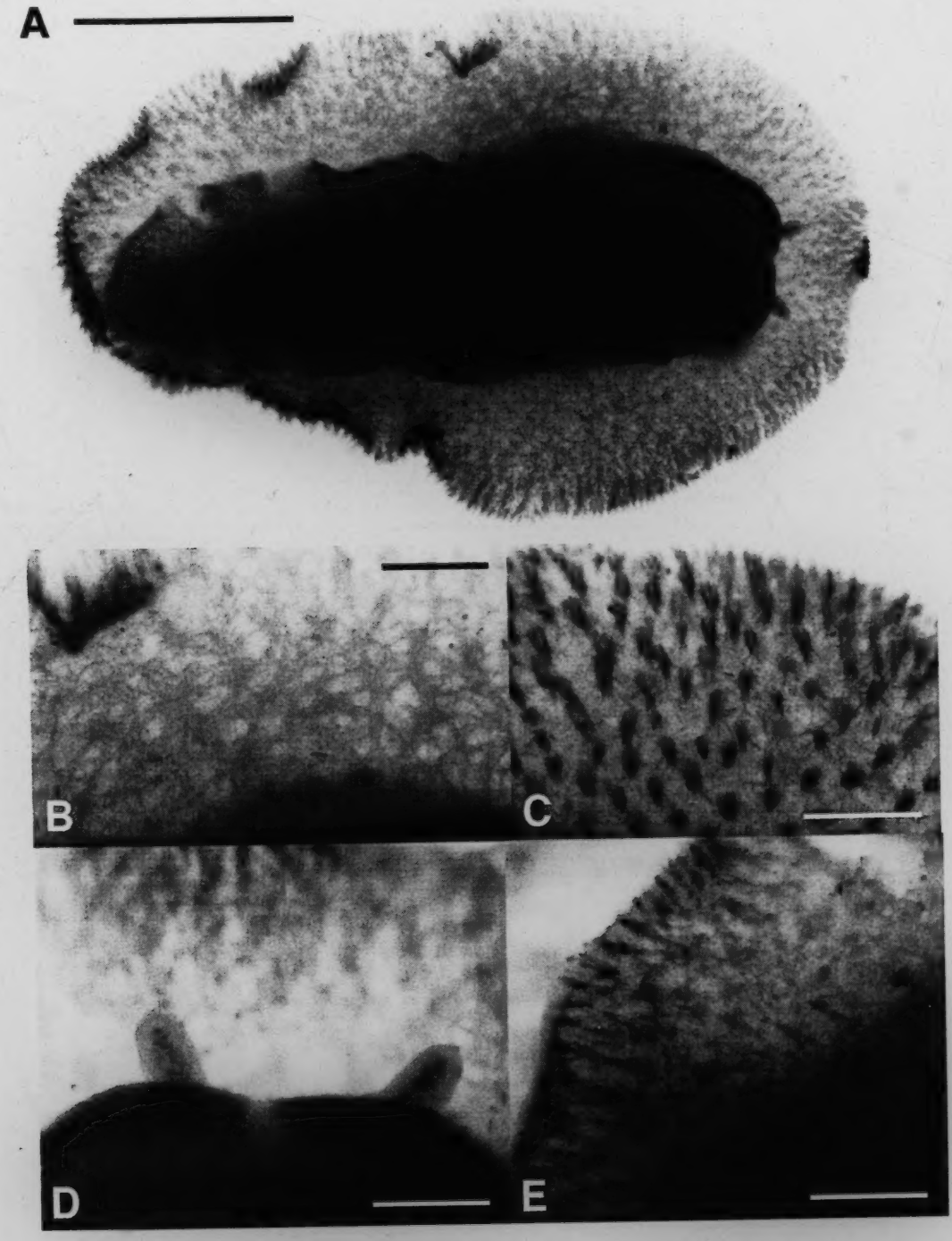
A. Ventral view of the whole specimen. Bar = 2.5 mm.

B. Ventral view of the mantle near the body wall, on the right side near the head. Bar = 0.5 mm.

C. Dorsal view of the mantle to show caryophyllidia. Bar = 0.5 mm.

D. Ventral view of the oral tentacles. The anterior of the specimen is to the top of the panel. Bar = 0.5 mm.

E. Ventral view of the foot edge, at the posterior end of the animal. Bar = 0.5 mm.


A
B
C
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E

**Figure 5-8.** Spicule networks of Sclerodoris coriacea mantle. The anterior side of the specimen is to the right in all panels.

A. Ventral view of the mantle, with the foot and viscera removed. Bar = 5 mm.

B. Ventral view of the central notum. Higher magnification of the area left of center in A. Bar = 2 mm.

C. Dorsal view of carophyllidia on mantle edge, at the posterior end of the specimen. Bar = 0.5 mm.

D. Ventral view of the mantle edge near the body wall to show detail of the spicule network. Bar = 0.5 mm.

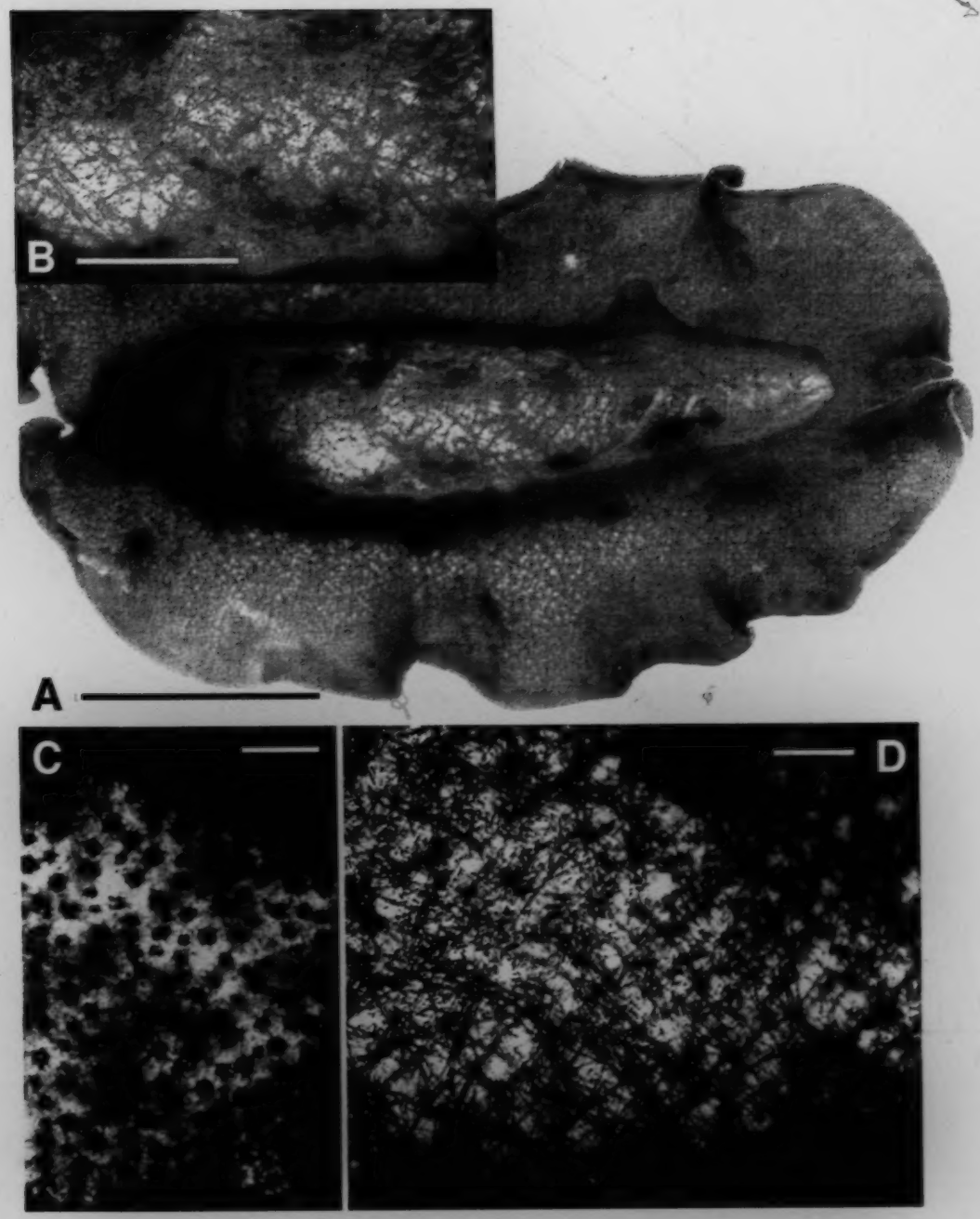
B
A
C
D

**Figure 5-9.** Spicule networks of Sclerodoris coriacea foot. The anterior side of the specimen is to the right in all panels, except D.

A. Dorsal view of the foot, with the mantle and viscera removed. Bar = 5 mm.

B. Dorsal view of the foot midline region. Magnification of the area near the center of panel A. Bar = 2 mm.

C. Higher magnification image of the foot midline region from B. Bar = 1 mm.

D. Dorsal view of the foot edge near the midline of the body. The anterior of the specimen is to the top. Bar = 1 mm.

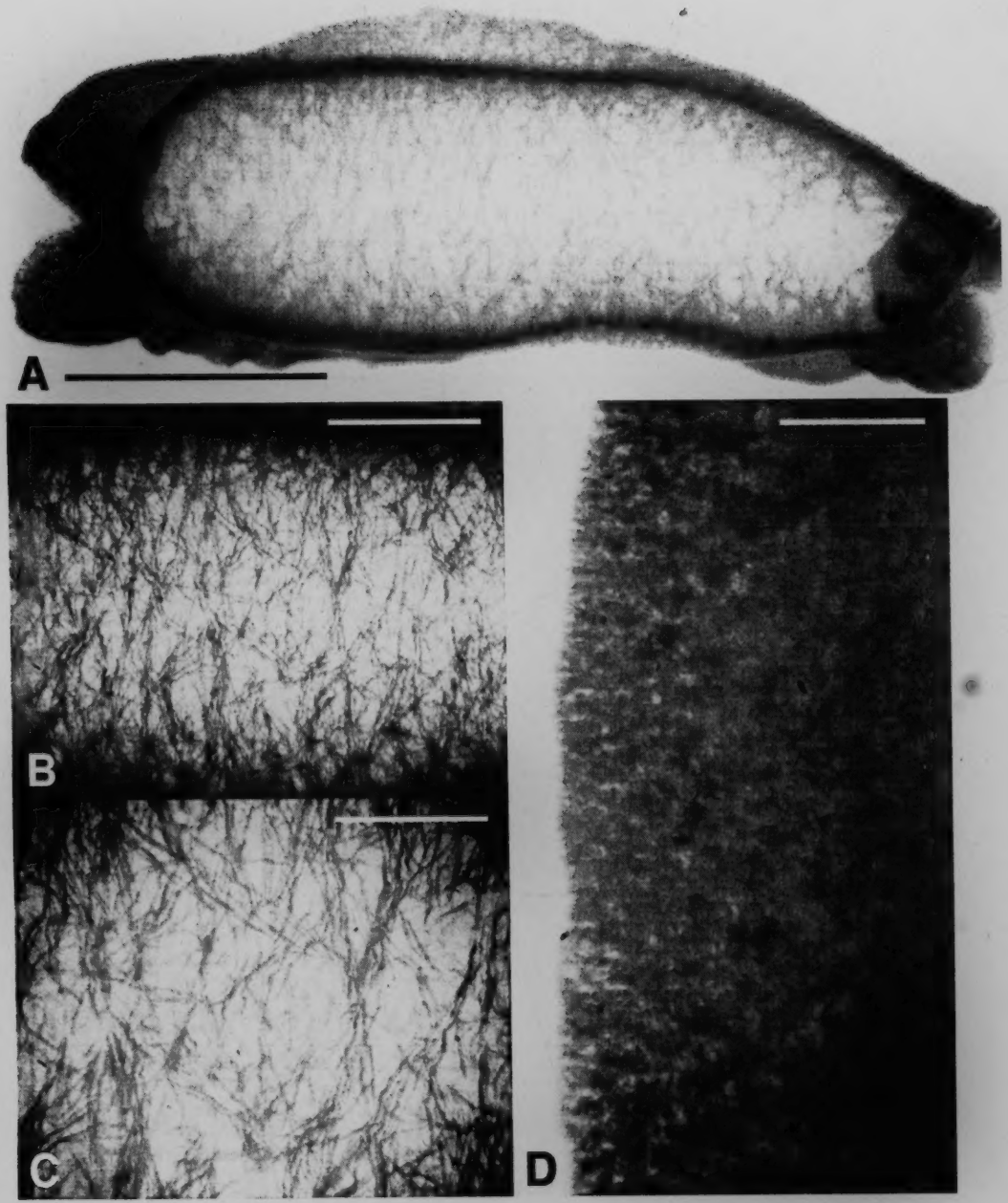
A
B
C
D

**Figure 5-10.** Spicule networks of Doriopsilla albopunctata. The anterior side of the specimen is to the right in all panels.

A. Ventral view of the mantle, with the foot and viscera removed. Bar = 5 mm.

B. Central notum, higher magnification of the region in the center of A. Bar = 2 mm.

C. Higher magnification of the mantle edge near the bottom center of A. Bar = 0.5 mm.

D. Dorsal view of the mantle surface to show papillae. Bar = 0.5 mm.

E. Dorsal view of foot, with the mantle and viscera removed. Bar = 5 mm.

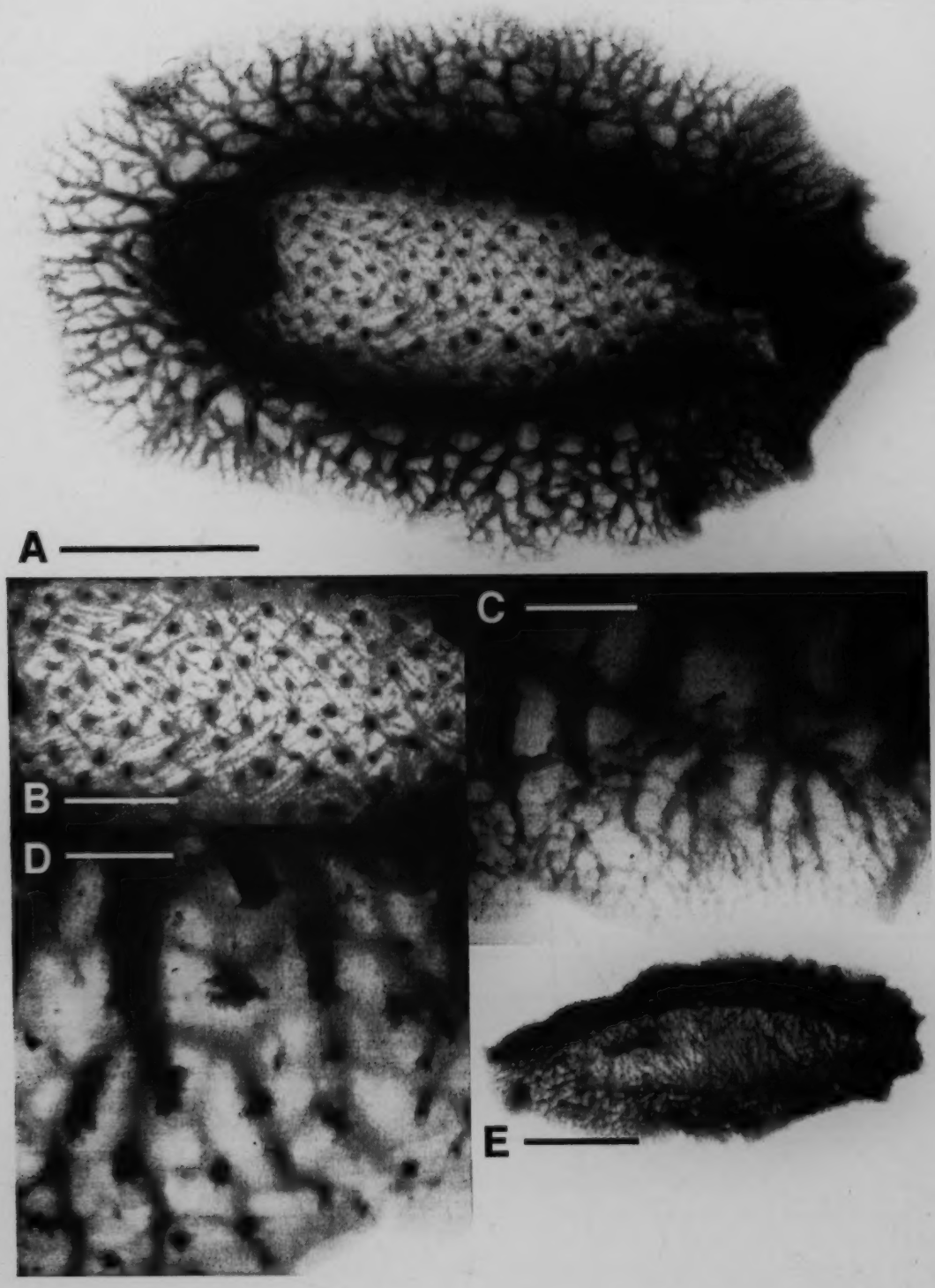
A
B
C
D
E

**Figure 5-11.** Spicule networks of Phyllidia varicosa. The anterior side of the specimen is to the right in all panels.

A. Dorsal view of mantle, with the foot and viscera removed. Bar = 5 mm.

B. Dorsal view of mantle edge near the middle of the body. Higher magnification of the central area near the top of A. Bar = 5 mm.

C. Dorsal view of papillae on mantle surface, with spicules lighter than the surrounding tissue before the specimen had been treated for overstaining. Bar = 1 mm.

D. Ventral view of the notum center. Bar = 1 mm.

E. Mid-section of the foot, with the mantle and viscera removed., in dorsal view. Bar = 1 mm.

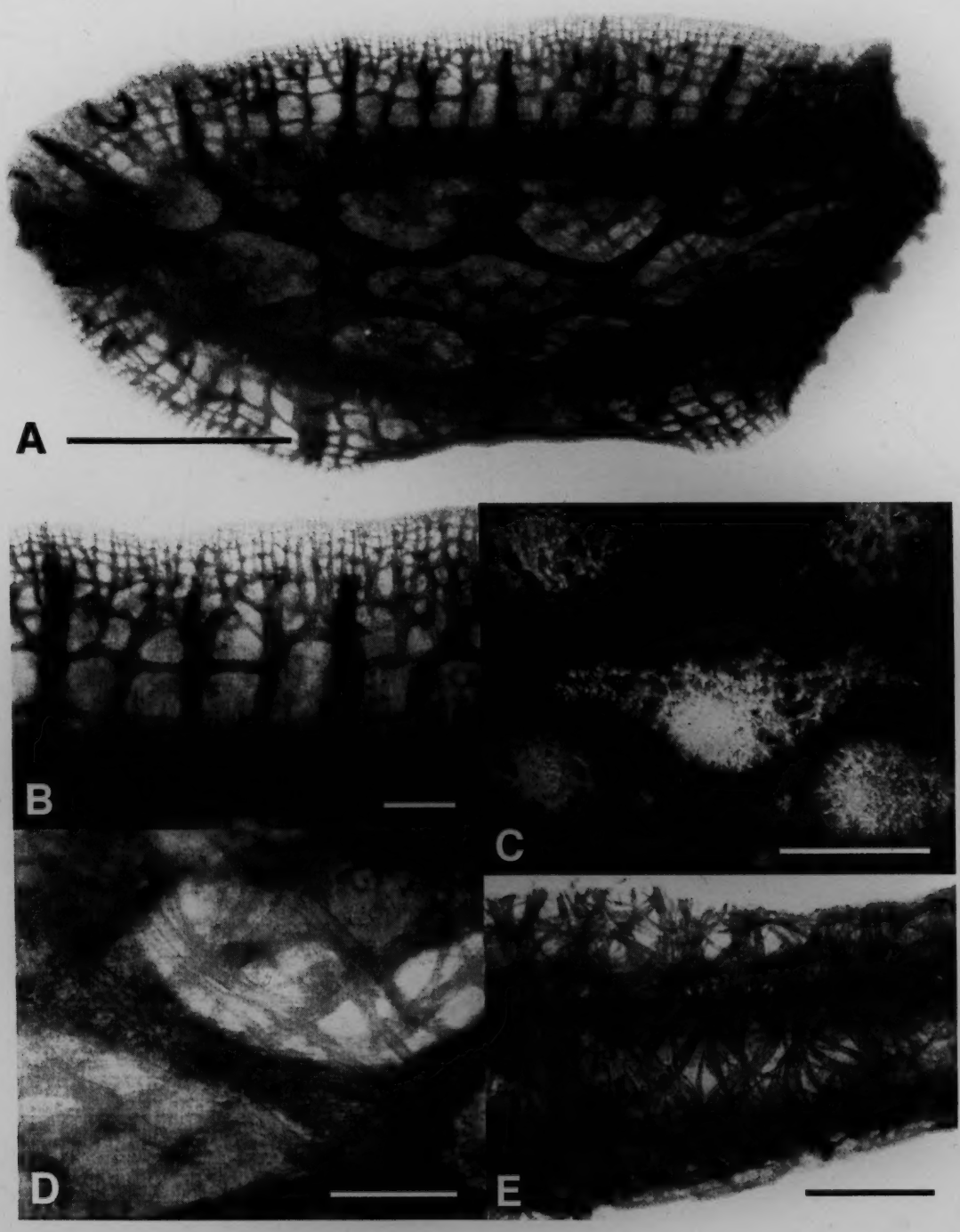
A
B
C
D
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**Figure 5-12.** Spicule networks of Phyllidiopsis cardinalis. The anterior side of the specimen is to the right in all panels..

A. Dorsal view of the mantle, with the foot and viscera removed. Bar = 5 mm.

B. Higher magnification of the right side mantle edge from right of bottom center in panel A. Bar = 1 mm.

C. Side view of one papilla on the notum. The dorsal surface is to the top of the image. Bar = 0.5 mm.

D. Higher magnification of the dorsal central notum, enlarged from the center of A. Bar = 1 mm.

E. Dorsal view of the central region of the foot. Bar = 1 mm.

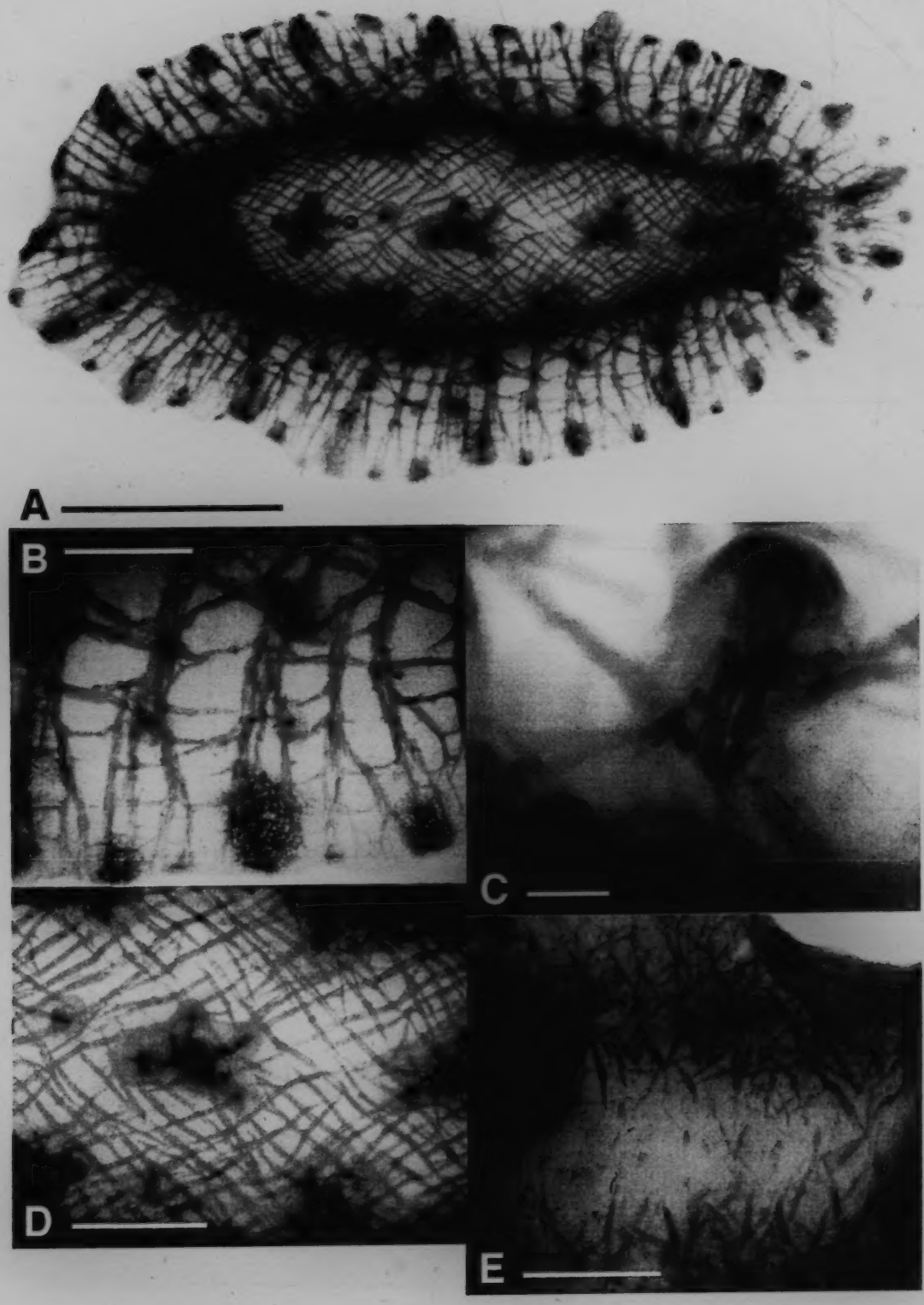

A
B
C
D
E

**Figure 5-13.** Spicule networks of Archidoris montereyensis. The dorsal surface of the specimen is to the top in all panels, except D.

A. Cross section of the mantle from near the midline of the body. Papillae on the dorsal surface are seen at the top of the image. Bar = 1 mm.

B. Higher magnification of the spicule network seen left of center at the bottom of A. Bar = 0.5 mm.

C. Cross section of the mantle, showing the spicule arrangement in papillae and their integration into the underlying mantle spicule network (arrow). Bar = 0.5 mm.

D. Dorsal view of the foot edge. Anterior is to the right. Bar = 5 mm.

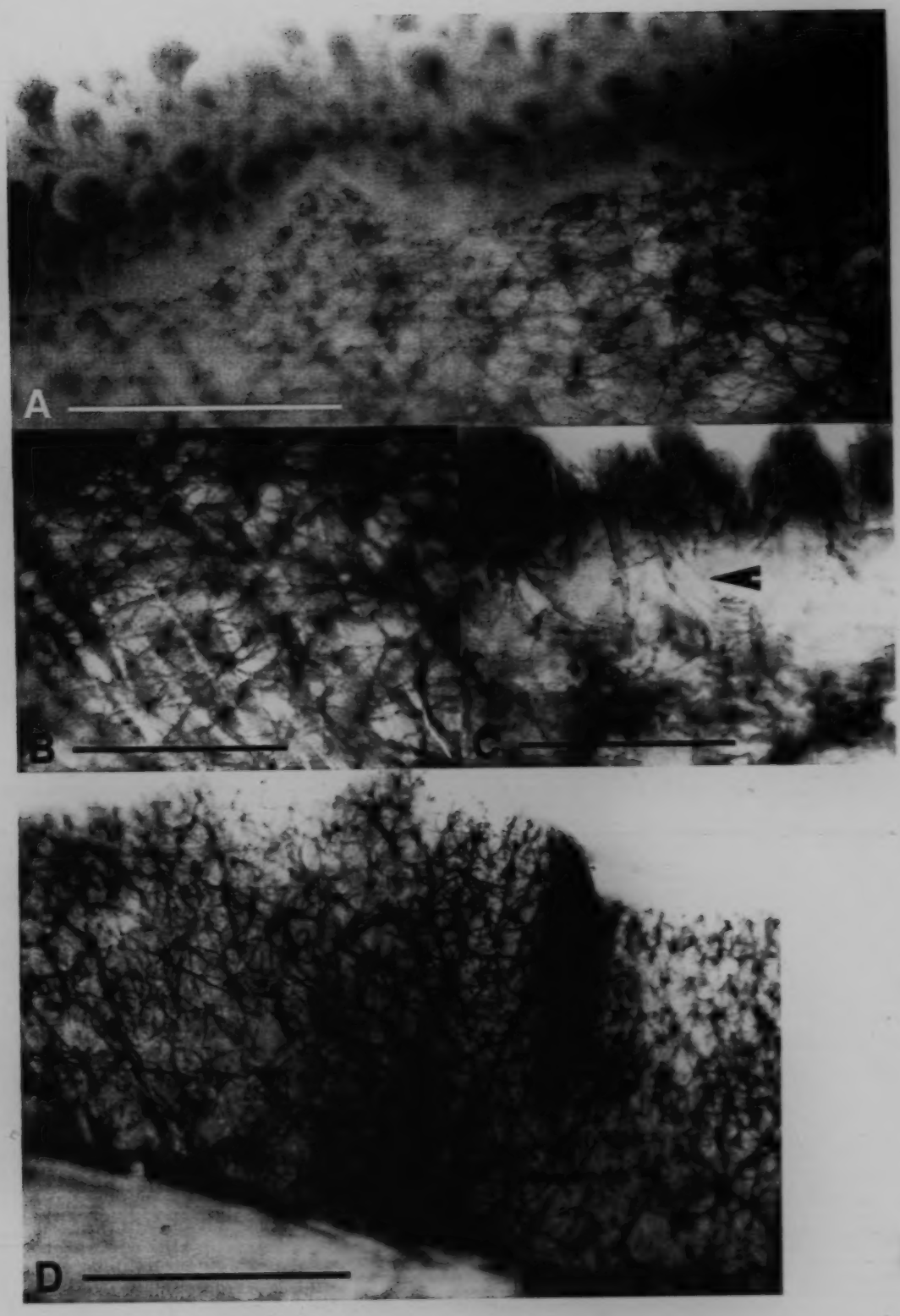

A
B
C
D

**Figure 5-14.** Spicule networks of Anisodoris nobilis.

A. Ventral view of the mantle edge near the middle of the body, with the anterior-posterior axis horizontal. Bar = 0.5 mm.

B. Dorsal surface view of papillae. The dorsal surface is to the top of the image. Bar = 1 mm.

C. Ventral view of the center and edge of the foot. Anterior is to the right. Bar = 5 mm.

D. Ventral view of the anterior lip of the foot. Anterior is to the right. Bar = 0.5 mm.

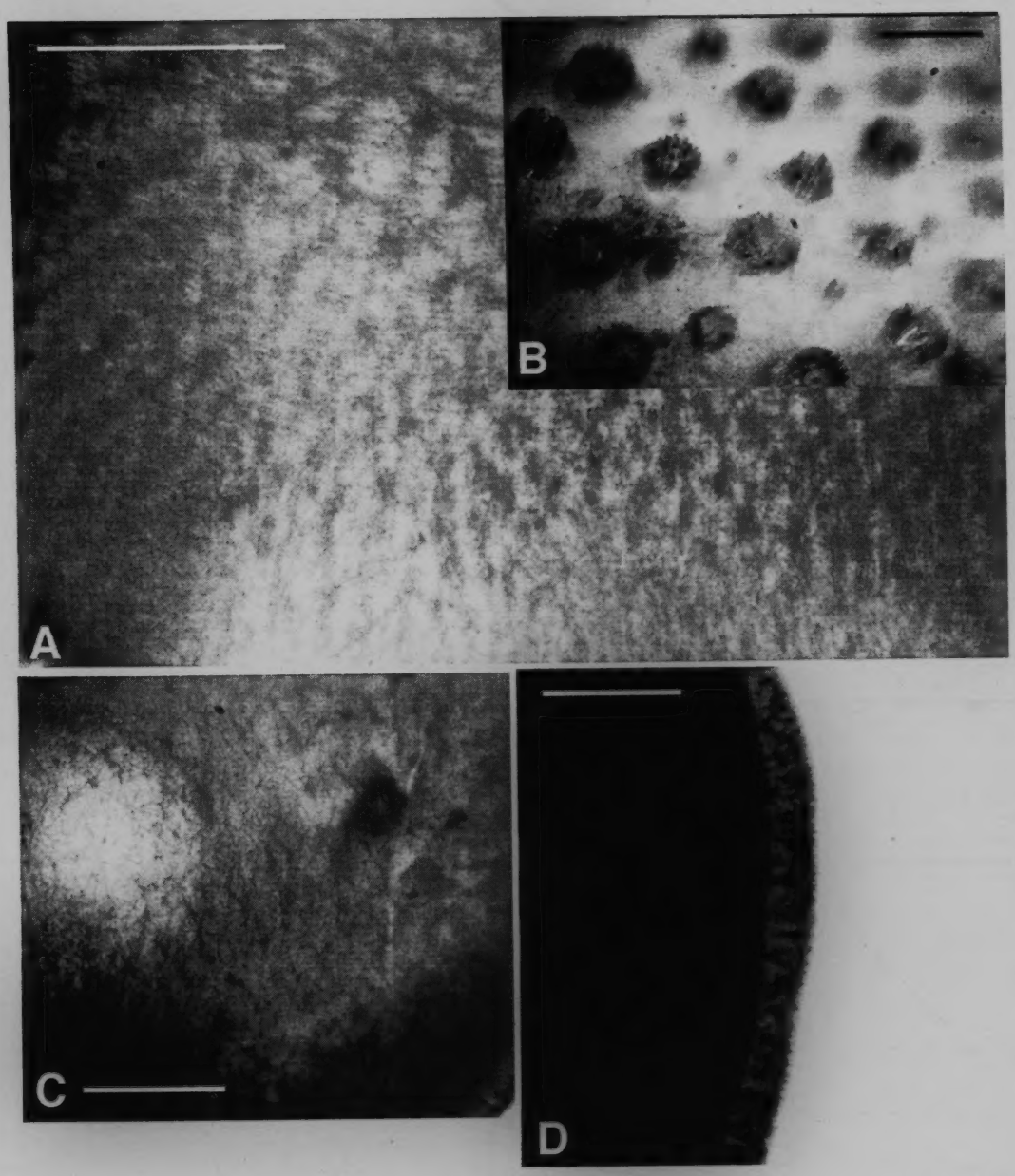

A
B
C
D

**Figure 5-15.** Spicule networks of Aldisa sanguinea. The anterior side of the specimen is to the right in all panels.

A. Ventral view of the mantle, with foot and viscera removed. Bar = 2.5 mm.

B. Dorsal view of the mantle edge. Bar = 0.5 mm.

C. Higher magnification of the mantle edge from the left side of A. Bar = 0.5 mm.

D. Dorsal view of the posterior foot. Bar = 2.5 mm.

E. Posterior edge of the foot. Bar = 1 mm.

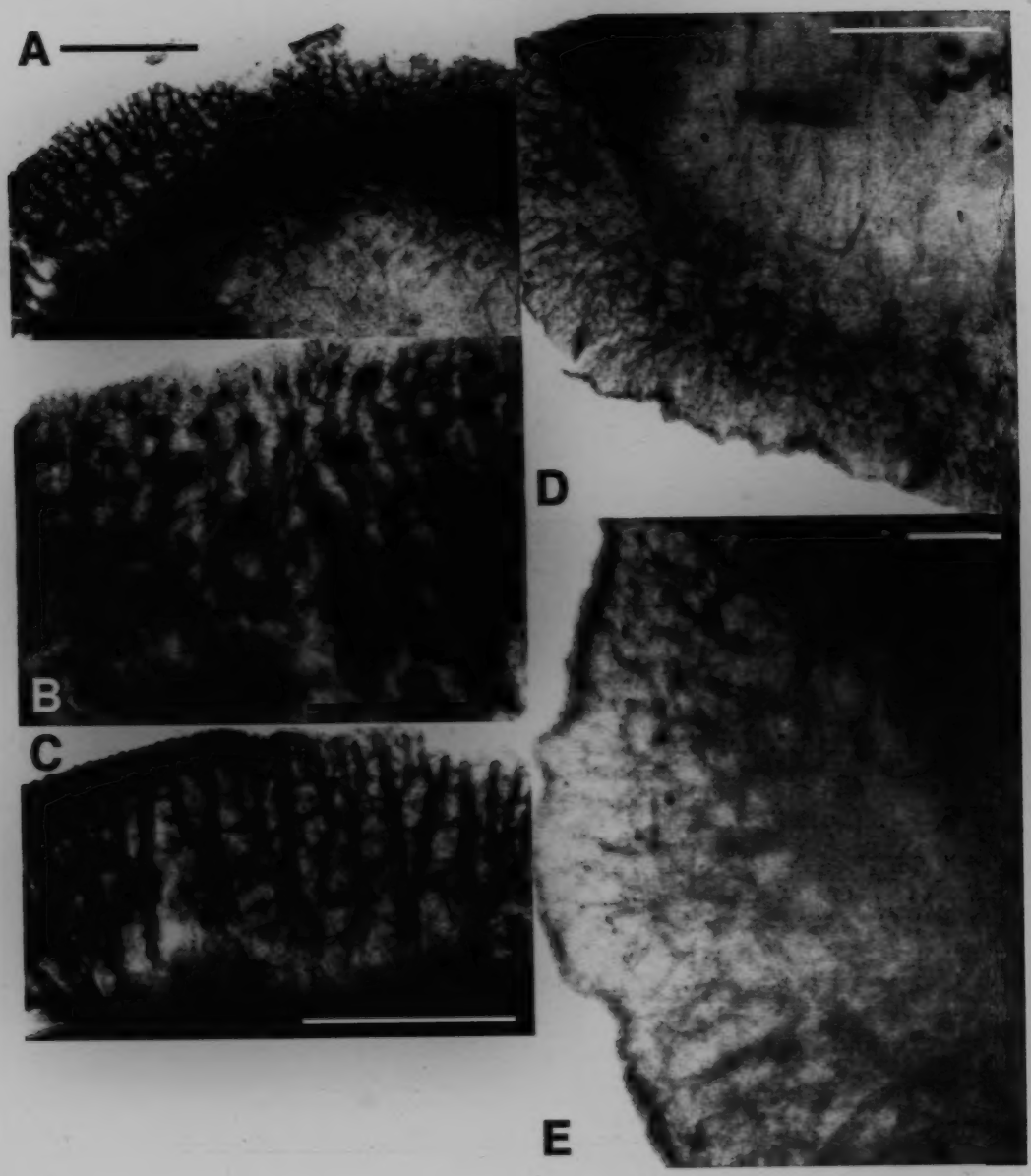
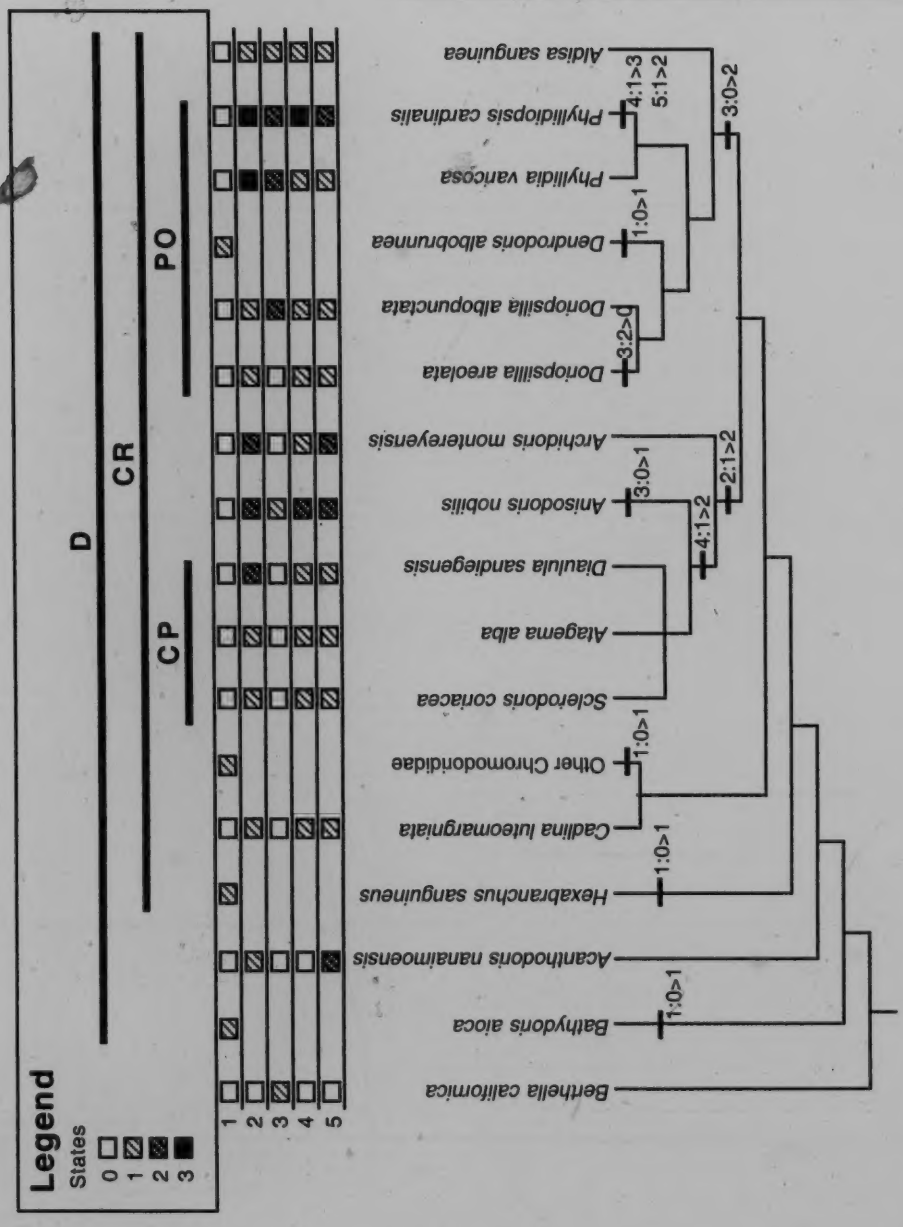

A
B
C
D
E

**Figure 5-16.** Mapping of spicule characters onto a preliminary phylogeny of the Cryptobranchia. Preliminary phylogeny combined from several published sources (Valdés and Gosliner 1999, Wägele and Willan 2000, Valdés and Gosliner 2001) with polytomies resolved using the spicule network characters discussed in the text. Horizontal lines in the legend indicate membership in described monophyletic groups: D, Doridacea; CR, Cryptobranchia; CP, caryophyllidia-bearer; PO, Porostomata. Data boxes indicate states of characters as described in the text.


Legend
States
0
1
2
3
D
CR
PO
CP
1
2
3
4
5
Aldisa sanguinea
Phyllidiopsis cardinalis
Phyllidia varicosa
Dendrodoris albobrunnea
Doriopsilla albopunctata
Doriopsilla areolata
Archidoris montereyensis
Anisodoris nobilis
Diaulula sandiegensis
Atagema alba
Sclerodoris coriacea
Other Chromodorididae
Cadlina luteomarginata
Hexabranchus sanguineus
Acanthodoris nanaimoensis
Bathydoris aioca
Bertheila californica
4:1>3
5:1>2
3:0>2
1:0>1
3:2>1
3:0>1
2:1>2
4:1>2
1:0>1
1:0>1
1:0>1

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# CHAPTER 6

## GENERAL CONCLUSIONS

**Feeding specialization in a marine context.**

Current evidence suggests marine communities are much less dominated by feeding specialists than are terrestrial communities (Lubchenco and Gaines 1981, Duffy and Hay 2001, Paul et al. 2001). The most typical pattern is for small, sedentary grazers to preferentially use marine plants providing chemical defense as shelter and/or food (Hay and Fenical 1988, Duffy and Hay 2001, Stachowicz 2001). However, while most studies have understandably focused on plants, many marine habitats are dominated by sessile, colonial invertebrates (Nybakken 2001). Similar studies on defense and prey use among carnivorous marine grazers could provide a useful test of hypotheses derived using phytophagous consumers. Of these grazers, nudibranchs are one of the most-extensively studied taxa, making them a promising system for study (Karuso 1987, Hay and Fenical 1988).

**How reliant are slugs on sequestered chemical defense?**

Nudibranchs, and dorids in particular, seem well defended against generalist predators (Todd 1981), although this has not often been directly tested (Faulkner 1992). The few tests of whole animals against realistic predators indicate a high level of rejection (Thompson 1960, Appendix II) and survival post-attack is high for at least one nudibranch (Appendix II). Given the lmited data on predation in natural

settings (Todd 1981), and reports of nudibranchs taken as prey by some predators (e.g., Birkeland 1974) it is unclear how far this generalization extends.

The nudibranchs from this study do not seem to rely solely on sequestered chemical defense. The level of deterrence seen from whole nudibranchs is often higher than that from specific compounds, suggesting that nudibranch defense is due to more than secondary chemicals (Faulkner 1992). Differences in nutritional quality, comparable to the minimum observed differences between slugs and snails, deterred some predators even without the addition of chemical extracts (Chapter 2). Given that nudibranchs are often rarer than other potential prey in the Northeastern Pacific (B. Penney, personal observation), and predators forage to maximize energy gained per unit handling time, nudibranchs are unlikely to be often targeted as food. Calcareous spicules have also been proposed as an extra line of defense for nudibranchs (Todd 1981, Garcia et al. 1986), and slightly increased the deterrence of chemical extracts against anemones, but not crabs (Chapter 3). Whether spicules are more deterrent to specialist predators of opisthobranchs (Paine 1963) remains to be tested. Lastly, the chemical defenses of many common Northeastern Pacific cryptobranchs are biosynthesized, not sequestered (Avila 1995, Kubanek et al. 2000). Indeed, slugs may not be restricted to one source of deterrent chemistry. Cadlina luteomarginata sequesters some metabolites while synthesizing others, and some evidence suggests that it regulates its production of endogenous chemicals based on what is available in the environment (Kubanek et al. 2000). Furthermore, while the slugs may derive additional benefit from close association with sponge prey (Anderson 1971, Gosliner

1985), they seem fairly well defended by endogenous defenses and the need for sequestered chemicals is unlikely a driving force in their diets.

**How specialized are these slugs?**

The two nudibranchs examined in this study (Anisodoris nobilis and Cadlina luteomarginata) were selective feeders in that they avoided the most common sponges at each site and, instead, consumed sponges that were relatively rare (Chapter 4). This pattern of feeding is similar to that found in other studies on this taxon: usually 7-10 sponges in a slug's diet at a given site, with 2-3 species comprising 70% of the total diet (Bloom 1981, Hellou et al. 1982, Thompson et al. 1982). However, the actual prey species used shift geographically, perhaps as a function of prey availability. There were few differences in diet composition despite different prey availability among sites within a few kilometers of each other (Chapter 4), but the differences in sponge community composition over a nudibranch's geographic range are likely more substantial. Over this range, slug populations may also be adapted to use different prey. Such differential adaptations of populations to locally available plants are seen in some sacoglossans (Trowbridge 1991) and decorator crabs (Stachowicz and Hay 2000).

Are these nudibranchs feeding "specialists"? The definition of feeding specialization is, to some degree, in the eye of the beholder: some authors include only monophagous species, whereas others include species feeding on a wide variety of species within a larger taxon (Futuyma and Moreno 1988). Although a strictly spongivorous diet is certainly a form of specialization (McClintock 1987), these

nudibranchs show a very different range of diet breadths than is seen in other purported "specialists." The most common slugs have broad diets comprised of sponges that are not often closely related (McDonald and Nybakken 1997). In contrast, over 80% of North American butterflies feed only on members of one plant family (Ehrlich and Murphy 1988). Sacoglossans – shell-less, herbivorous gastropods – show a very similar pattern to phytophagous insects, whereas many other marine herbivores will eat plants from ten or more families (Hay 1992). The degree of specialization in nudibranchs may be closest to that seen in cone snails (Conus spp., see Duda et al. 2001, for review). Most of these snails feed on prey from one or two phyla, often ones that are morphologically similar, although many consume only one or a few families of prey.

**How has diet-breadth evolved in this group?**

Experimental data for these dorid nudibranchs do not support the need for sequestered chemical defense as a driving force for specialized diets. Many slugs have other endogenous defenses and, perhaps predictably, broad diets. Also, the nudibranchs in this study are fairly large compared to other "mesograzers" and typically obliterate patches of their prey (Bloom 1974) rather than staying on them as habitat. Feeding specialization may also be facilitated by brooding in crustaceans (Hay and Fenical 1988), and the slugs in this study have planktonic larvae (Strathmann 1987). Lastly, behavioral facilitation of host switching may be important for phytophagous insects (Feeny 1991). Although some evidence exists that specific chemical cues are important for prey location by dorids (Appendix I, Carté and

Faulkner 1986) we currently know too little about slug chemotaxis to test this hypothesis (Faulkner 1992).

To what degree can we generalize these results to other cryptobranchs? Certainly, the more specialized species in this region (e.g., Rostanga pulchra, Aldisa sanguinea) were under-represented, because they are typically rarer. Both species are smaller than those included in this thesis, and known to either sequester (Anderson 1971) or modify metabolites from their prey (Ayer and Andersen 1982). Both also have few reported prey species, suggesting body size and sequestration may influence diet breadth. Interestingly, one of the larger cryptobranchs, Hexabranchus sanguineus, modifies chemicals obtained from its prey (Pawlik et al. 1988) but is a very nonspecific grazer, perhaps even eating other sessile colonial invertebrates (Francis 1980). Some Antarctic slugs are essentially sponge generalists, only avoiding the most toxic species (McClintock 1987). The Chromodorididae seem more specialized than other taxa (McDonald and Nybakken 1997), but also have a lower reported incidence of biosynthesis (Avila 1995). However, they are also restricted to warm temperate or tropical waters (Rudman 1984), where predation pressure is likely higher. Given the confounding effects of geography, taxonomic similarity, and other factors, the most useful tool to test hypotheses of feeding specialization will be a robust phylogeny for this group. Recent studies have allowed rigorous testing of these scenarios for phytophagous insects (Becerra 1997, Kelley and Farrell 1998), but require better phylogenetic characters and data on prey use for nudibranchs.

**Cryptobranch phylogeny**

Tests of phylogenetic hypotheses require robust phylogenies, and these have been hard to obtain for nudibranchs, partially because of their lack of characters that can be readily measured (Foale and Willan 1987, Valdés and Gosliner 2001). Unfortunately, attempts at molecular systematics with this group have proven equally confusing (Thollesson 1999a,b). Some progress has been made recently with the adoption of new morphological characters, including electron microscopical characterization of nudibranch morphology (Kress 1981, Foale and Willan 1987, Wägele and Willan 2000, Valdés and Gosliner 2001). However, such studies are expensive and equipment-intensive. However, spicule networks occur in many cryptobranchs, and are easily and inexpensively examined, and seem to provide sufficiently clear and consistent variation for use in phylogenetic systematics (Chapter 5). Mantle networks show a distinct gradient from haphazard arrangments to either distinct radial and circumferential tracts or a finer, cobweb-like arrangement. Spicule arrangment through the papillae has helped resolve the relatedness of some taxa, indicating all caryophyllidia-bearing dorids seem closely related (Valdés and Gosliner 2001). Other features are promising, including networks in gills and rhinophores (Chapter 4) or through the foot or along its border (Chapter 5). Some characters, such as spicule presence, show obvious reversals and parallelism. However, this is a problem for most opisthobranch taxa (Gosliner 1985). Other morphological characters show some variation, such as brain morphology (Valdés and Gosliner 2001), but these traits vary too much within taxa to be useful in higher-order taxonomy.

**General significance**

The topology suggested by spicule networks and other morphological characters suggests multiple transitions between specialist and generalist sponge consumers within the Cryptobranchia. Hexabranchus sanguineus may be one of the least-derived cryptobranchs, and has a very non-specific diet (Francis, 1980, but see Pawlik et al. 1988). Diaulula and Rostanga are sister genera (Valdés and Gosliner 2001), yet Diaulula sandiegensis has a far more catholic diet than Rostanga pulchra (McDonald and Nybakken 1997). Likewise, the Chromodorididae are one of the most conspicuously specialized taxa, yet Cadlina luteomarginata has one of the broadest diets known for a cryptobranch (McDonald and Nybakken 1997). However, Aldisa sanguinea has a specialized diet, despite possibly being basal to the more specialized porostomes or other taxa. Although this group likely shows a progression from less- to more-specialized (Cimino and Ghiselin 1999), diets have certainly expanded and contracted along all lineages.

What characters correlate with diet-breadth? Sequestered chemicals may be important (Faulkner and Ghiselin 1983, Cimino and Ghiselin 1999). While many slugs possess alternate physical defenses against consumption (Chapters 2,3), these are probably not alone sufficient to protect the nudibranchs. We lack critical evidence on biosynthesized chemistry for several groups (Avila 1995), but certainly the species that sequester defenses are among the most conspicuous feeding specialists. Body size and dispersal relative to prey may also play a role in selection for feeding specialists: sacoglossans and other specialist taxa often have smaller brood sizes, and

sometimes direct development (Hay and Fenical 1988). Within the cryptobranchs, Cadlina laevis is long-lived compared to congeners, iteroparous and direct developing (Todd et al. 2001); it also has a narrower diet than C. luteomarginata and other congeners (McDonald and Nybakken 1997) that have planktonic larvae. Also, carnivores may be less specialized than herbivores, although further work is required to clarify whether nudibranchs have a closer level of specialization to Conus snails or to their (comparatively) near relatives, the sacoglossans.

In the end, sea slugs may yet provide a good system by which to test hypotheses of feeding specialization based on herbivorous insects (Karuso 1987). The importance of determining what factors drive consumers to use only particular prey have already been stated (Chapter 1). However, restriction of one niche aspect may correlate with restriction elsewhere (Futuyma and Moreno 1988). Species that are comparatively restricted in their habitat requirements are also more likely to go extinct by random factors (Futuyma 1998). Given the rate at which we are degrading near-shore marine habitats (Nybakken 2001), we may be losing one of the more conspicuous and attractive parts of our marine fauna.

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# APPENDIX I

## NONPOLAR COMPONENTS OF PREY SPONGE EXTRACT ATTRACT ROSTANGA PULCHRA (NUDIBRANCHIA:DORIDACEA)[2]

### Introduction

Opisthobranchs which lack the physical protection of a shell require other antipredator defenses such as noxious compounds or crypsis (Thompson 1976). Rostanga pulchra MacFarland 1905 mimics the color of its sponge prey almost perfectly by sequestering carotenoid pigments in the same proportions as found in several prey sponges (Ophlitaspongia pennata, Esperiopsis originalis, and Plocamia karykina (Anderson 1971)). Yet R. pulchra often moves between patches of sponge and feeds on several different species (Anderson 1971); how does a slug find sponges containing the compounds needed for crypsis?

Chemoreception is important for gastropods in general (Sakata 1989) and several dorids can use chemotaxis to locate their prey (Cook 1962, Anderson 1971, Elvin 1976). It would seem adaptive if nudibranchs were attracted to prey via the same compounds they sequester. However, although most sequestered compounds are nonpolar (Avila 1995), nonpolar attractants are only known for herbivorous gastropods; no attractive nonpolar prey compounds are known for carnivorous gastropods (Kohn 1961, Audesirk and Audesirk 1985, Sakata 1989).

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[2] A version of this appendix has been published as Ong and Penney 2001 Veliger 44: 99-100

Therefore we used a Y- maze design to test whether R. pulchra is attracted to:

a) whole sponges

b) sponge extracts (dissociated compounds)

c) the nonpolar fraction of sponge extracts, which includes carotenoids

**Methods**

Collections

Thirty R. pulchra from 4 to 15 mm long and several rocks with O. pennata were collected intertidally or by SCUBA from Barkley Sound, British Columbia in October and November 1998, and kept in running natural seawater at Bamfield Marine Station. Nudibranchs were kept in individual mesh-sided containers upstream from rocks with sponges, and fasted for 6-18 days before being tested.

Extracts

Sponges were scraped from rocks and thrice extracted with three times volume of methanol for 24 hrs each time. This extract was divided in two, half for the whole extract assays, and half for the nonpolar extract assays. The latter fraction was extracted in a separatory funnel three times with an equal volume of hexane, and all three hexane portions combined as "nonpolar extract". Both extracts were reduced to 2.5 ml in a rotary evaporator at ≤35°C. For the assays, whole extracts were contained in 7% agar blocks, but 8.5% agar was required for hexane extracts and controls to solidify.

Y-Maze Assays

We assayed responses of R. pulchra in a Y-maze of clear, nonporous plexiglass (36 x 10 cm, with arms 21.5 x 10 cm). Flows were balanced between arms using dyes, and were equivalent for each test. Treatments for each assay (sponge, whole extract, nonpolar extract) were randomly assigned to arms for each assay, and seawater run through the apparatus for several minutes to allow compounds to equilibrate. Nudibranchs were then placed in the middle of the Y maze and allowed to crawl freely. We recorded a 'choice' if the nudibranch progressed more than two body lengths down either arm, and 'no choice' if it had not entered either arm after 30 minutes. The Y-maze was emptied of water and scrubbed to remove mucus trails after each trial. Each nudibranch was tested once against sponges, whole extract, and nonpolar extract sequentially; we feel this nonrandom order did not affect the significance of the results. To make our statistics conservative, animals dying before completion of all tests were excluded from analysis, and 'no choice' animals were counted as having chosen the control.

**Results**

Approximately 80% of the nudibranchs chose the treatment arm in each experiment. The stimulus position significantly affected which arm the slugs chose (Table I-1). The slugs also preferred the left arm of the maze, possibly due to slight differences in the flow rate. Allocation of treatments to each arm was roughly 50%.

**Discussion**

R. pulchra is attracted to whole O. pennata, confirming the results of Cook (1962) and Anderson (1971). In Anderson's assays, it is interesting that R. pulchra was not attracted to E. originalis, another prey sponge containing the right mix of carotenoids; this may be due to the motivation of the animals, or the arena she used.

R. pulchra also responds to isolated compounds from this sponge, suggesting that chemotaxis is an important means of prey location for this nudibranch. It responds to nonpolar compounds at a level equivalent to that for whole extracts; this is the first report of nonpolar attractants for a carnivorous gastropod. Determining whether the actual nonpolar attractants are the carotenoids sequestered by the nudibranchs bears more investigation, as other nudibranchs also sequester carotenoids or other pigments (Anderson 1971). Regardless, nonpolar compounds are often used by nudibranchs for defense (Avila 1995), whether for coloration or as toxins, suggesting adaptation to detect at a distance the compounds sequestered from prey.

**Table I-1.** Responses of Rostanga pulchra to Ophlitaspongia pennata treatments in a Y-maze. Treatment vehicles were rocks for sponges and agar blocks for extracts; controls for each treatment were bare rocks or agar blocks with solvent only. Significant differences in slug position were tested via $X^2$ tests with continuity correction. See methods section for details.

| | | Slug position | | | |
|---|---|---|---|---|---|
| Treatment | Stimulus position | Left | Right | $X^2$ | P |
| Whole sponge | Left | 10 | 1 | 5.860 | 0.016 |
| | Right | 3 | 7 | | |
| Sponge Extract | Left | 11 | 2 | 4.868 | 0.027 |
| | Right | 4 | 8 | | |
| Nonpolar Fraction | Left | 12 | 0 | 7.106 | 0.008 |
| | Right | 4 | 6 | | |

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# APPENDIX II.

## WHOLE ANIMAL ANTIPREDATOR DETERRENCE AND POST-ATTACK SURVIVAL FOR CADLINA LUTEOMARGINATA (NUDIBRANCHIA: DORIDACEA)

### Introduction

Most nudibranchs seem well-defended against consumption by predators (Harris 1973, Thompson 1976, Todd 1981) through secondary chemicals (Avila 1995), lowered nutritional quality (Chapter 2) and, to some degree, calcareous spicules (Chapter 3). Many sea slugs also possess means to avoid detection and attack by predators, such as crypsis or aposematism (Thompson 1976, Gosliner and Behrens 1990). However, defense against consumers is always context-dependent (Belovsky and Schmitz 1994); most recent studies of nudibranch defense have used laboratory assays with single types of defense or secondary compounds against model "predators," and older studies present mostly anecdotal findings against one type of predator (Thompson 1960). While such reductionist studies are definitely useful, they can complicate extrapolations to how nudibranch defenses function in natural environments.

First, are these chemicals and other defenses effective against the predators actually encountered in the field? Predators certainly vary in their response to defenses (Rogers and Paul 1991, Cronin and Hay 1996), and most studies have used goldfish or other predators of dubious relevance to natural settings (Hay 1996). It is unclear what predators might be appropriate: some birds, fish, sea stars, and

specialized opisthobranchs eat some species of nudibranchs (Todd 1981), but the range of nudibranch species which they consume is unclear. One could argue about which predators are ecologically relevant: generalist predators that do not consume slugs could be deemed unimportant while specialist predators are adapted to slug defenses. However, because the highest predation pressure in most marine habitats is probably from abundant omnivorous generalists, the most reasonable test is against generalist predators found in the same habitat as the slugs (Hay 1996).

Second, because most lab assays use artificial food as substrate, we often have little idea how the slug itself is affected by attacks. Deterring predators is only directly advantageous if the nudibranch survives attacks well enough to reproduce again. Although some benefit might possibly be derived from kin selection, this seems unlikely for nudibranchs as they are often uncommon (Todd 1981) and have planktonic, widely dispersing larvae (Strathmann 1987). Thus, while chemical defense has been claimed as the driving force behind color patterns (Gosliner and Behrens 1990) and patterns of prey use (Cimino and Ghiselin 1999), such connections could be strengthened with evidence that chemical defense directly increases an individual slug's survival.

Both concerns can be addressed by experiments that test whole slugs against relevant consumers, and by observations of nudibranchs after exposure. Dorid nudibranchs are chemically the best-investigated taxon of nudibranchs (Avila 1995) and so make a reasonable study system. Therefore, I assayed a common dorid nudibranch, Cadlina luteomarginata, against several generalist predators from its habitat and recorded its survival post-attack.

**Methods**

Animal collection and maintenance

Cadlina luteomarginata is a common cryptobranchiate dorid nudibranch with known chemical defense (Avila 1995, and references therein). Individuals were obtained from Grappler Inlet, Bamfield, British Columbia by hand using snorkeling or SCUBA. In case chemically deterrent prey held in the digestive system can serve as a predator deterrent, slugs were maintained in the laboratory for ca. three days without food to clear their guts of sponge remnants but without losing sequestered compounds.

The most common generalist predators at sites where C. luteomarginata is abundant include large crabs (primarily Cancer), sea stars, fish, and anemones (B. Penney, unpublished data). I selected several of the most common molluscivorous or omnivorous species for bioassays. Cancer productus and C. gracilis (Crustacea: Decapoda) were collected by baited trap from Bamfield and Grappler inlets, Bamfield, B.C. Anemones, Anthopleura elegantissima (Cnidaria: Anthozoa), were collected by hand from Scott's Bay, Bamfield, B.C. The sun star, Pycnopodia helianthoides (Echinodermata: Asteroidea), a voracious predator that was common at all field sites, was collected by hand using SCUBA. Kelp greenling, Hexagrammos decagrammos (Ostheichthes) were caught by line and hook. All predators were fed mussels (Mytilus spp., ca. 4 cm long) every other day to train them to feed in the lab, after which they were fed 2 $cm^2$ pieces of squid mantle for approximately one week to

train them to receiving experimental food. This produced predators that were hungry, but not starving, for bioassays.

Bioassays and Survival

Predators were presented with both control food (squid mantle, ca. 2 $cm^2$) or one nudibranch, one at a time in random order. Individuals that did not sample either the control food or nudibranch (i.e., manipulating them with chelae or taking the food into their mouth) were excluded from analysis as being insufficiently motivated to feed. Predator consumption of food was recorded over a period appropriate to their mode of feeding: crabs and fish responses were recorded after 5 min, while anemones and sea stars were watched for 24 h. Prey were considered as consumed if they were swallowed and not spit out whole during the period of observation. Predators were also monitored for several days after the experiment to confirm slugs were not regurgitated. Data were analyzed using Cochran's Q test (Zar 1984).

After sampling by predators, nudibranchs were kept in 1 L mesh-sided cages in natural flowing seawater for one week, during which survival and slug condition were recorded daily. A few slugs escaped their cages during the observation period, and so were excluded from further consideration. All nudibranchs were then returned to the collection sites.

**Results**

Whole slugs were extremely deterrent to predators: over the course of the experiment, only one nudibranch was eaten and not spit out whole within 24 h (Table. B-1). This lone act of consumption was confirmed when the sea star regurgitated partially digested remnants of the nudibranch several days later. Most slugs (ca. 80%) survived at least one week after exposure to predators (Table II-1). However, the only slugs that died were those attacked by swallowing predators (75% survival) while no slugs died from clipping attacks.

**Discussion**

The overall low consumption of C. luteomarginata indicates it is effectively defended against several generalist predators from different phyla and with different feeding modes. Interestingly, predators rejected whole C. luteomarginata much more consistently than in previous experiments using food treated with only chemical extracts at natural concentrations (Chapter 3) or single compounds (Hellou et al. 1982, Thompson et al. 1982). This discrepancy could have several causes. First, the efficacy of chemical defense often varies with predator type (Belovsky and Schmitz 1994, Duffy and Hay 2001, Stachowicz 2001). While C. luteomarginata metabolites have been previously tested against tidepool sculpins (Oligocottus maculosus) (Thompson et al. 1982), other predators may vary in their response or we may not have found the full range of deterrent compounds for this nudibranch. Second, this nudibranch likely has other defenses against consumption by predators, including lowered nutritional quality (Chapter 2) and possibly spicules (Thompson 1976,

Chapter 3). Third, these defenses may interact synergistically (Duffy and Hay 2001, Stachowicz 2001). Overall, assays with whole animals or extracts, followed by bioassay-guided fractionation may provide a more ecologically relevant way to investigate defense (Faulkner 1992).

Survival of C. luteomarginata post-attack was often high (ca. 80%), but varied with mode of predator attack. No nudibranchs died after attacks by crabs – who clip and pierce the mantle edge with their chelae while sampling (B. Penney, pers obs.) – and the slugs' wounds began healing within the week. In contrast, survival was much lower (75%) for slugs attacked by swallowing predators such as anemones and sea stars. This suggests attacks by some predators are much more damaging than those of others. Indeed, nudibranchs regurgitated by anemones within 24 h were often partially digested, and did not survive long. It would be interesting to test whether nudibranch defenses are more or less effective against these types of predators, and the relative importance of these predators in the field.

How applicable are these results to other nudibranchs? While most nudibranchs are undoubtedly well-defended (Todd 1981), some species are consumed regularly by predators (e.g. Birkeland 1974) and which species are acceptable prey, and to which predators, is unclear. A large number of British opisthobranchs are deterrent to fish (Thompson 1960), but tests of whole slugs are generally rare. The applicability of existing laboratory assays using isolated compounds to natural settings are harder to assess. However, in years of observation in British Columbia, I have only seen one instance of predators attacking a nudibranch in the field: a crow (probably Corvus caurinus) pecking out the viscera of an Archidoris montereyensis

and leaving the exterior tissues. Few other data exist on nudibranch survival after attack, but if survival is consistently high in most cases, it would suggest a more plausible route by which the current patterns of chemical defense and coloration could have evolved in such sparsely distributed animals. Likewise, further tests of whole-nudibranch deterrence could provide a useful positive control against which to test the effectiveness of individual secondary metabolites.

**Table II-1**. Assays of Cadlina luteomarginata against generalist predators. Survival of nudibranchs to seven days post-attack. "Live" and "Die" categories indicate whether the nudibranchs survived attack by the predators. N for survival only includes nudibranchs monitored to the end of the experiment.

| | Consumed | | Survival | | | |
|---|---|---|---|---|---|---|
| **Predator** | **N** | **eaten** | **live** | **die** | **7 Days** | **N** |
| Cancer gracilis | 10 | 0 | 10 | 0 | 100 % | 10 |
| Cancer productus | 11 | 0 | 9 | 0 | 100 % | 9 |
| Hexagrammos decagrammos | 8 | 0 | 1 | 1 | 50 % | 2 |
| Pycnopodia helianthoides | 10 | 1 | 8 | 1 | 89 % | 9 |
| Anthopleura elegantissima | 9 | 0 | 6 | 3 | 67 % | 9 |

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